

Pharmacological Preconditioning of Human Hepatocytes

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Abstract

Liver surgery, be it for resection or transplantation, causes injury to the organ.

Pretreatment of the liver to protect it from injury would be of clinical interest as poor liver function following surgery can be life threatening. Such “preconditioning” treatments could be applied to both elective liver surgery and liver transplantation.

The aim of this project was to establish the ability of certain pharmacological agents to induce the stress response in human hepatocytes, and to protect them from the type of injury seen in surgical practice. Curcumin, an extract of the spice turmeric, was found to induce the enzyme heme oxygenase-1. It preconditioned hepatocytes to survive in models mimicking both ischaemia-reperfusion injury and the cold preservation injury which occurs during transplantation. Cyclosporin A was also studied as a putative preconditioning agent. However, although it did have some effect on the stress response, it did not precondition human hepatocytes. As an extension of the work on heme oxygenase 1, the effect of a promoter polymorphism in the human HO-1 gene was studied. This work has demonstrated that curcumin does pharmacologically precondition human hepatocytes *in vitro*. It has also strengthened the evidence for heme oxygenase 1 as a target for preconditioning strategies.

Declaration

I hereby declare that this thesis has been composed solely by myself and has not been submitted elsewhere for any other degree or professional qualification. All work presented in this thesis was, unless otherwise acknowledged, initiated and executed by myself. All sources of information in the text have been acknowledged by reference.

Stephen Justin McNally

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Chapter 1

General Introduction

1.1 Context

Liver transplantation is dependent on successful function of the implanted allograft. The process of organ harvesting, cold storage and reperfusion is damaging, and causes significant injury which can result in primary non-function or prejudice long-term graft survival. Primary non-function and initial poor function affects a large number of liver transplants (6% and 15% respectively), causing significant morbidity and mortality (Strasberg 1994). Pretreatment of the liver prior to its harvest to protect it from injury would be of great clinical importance. It may be life-saving, and could improve long-term graft survival. Similar damage occurs during elective liver surgery, resulting in perioperative hepatic impairment, a major contributor to morbidity and mortality following liver resection (Parks 2001). Pretreatment of the liver prior to surgery could reduce postoperative hepatic impairment and may improve clinical outcomes. Hence “preconditioning” treatments have a significant clinical potential in the area of liver surgery and transplantation (Selzner 2003).

1.2 Ischaemia Reperfusion Injury

Injury to the liver during elective surgery and transplantation is due predominantly to the attendant ischaemia-reperfusion injury which occurs as a result of the surgical process. Ischaemia reperfusion injury is, as its name suggests, a two stage phenomenon which comprises: damage sustained during an ischaemic, hypoxic period where blood inflow to the tissue is halted; and the subsequent damaging effects associated with restoration of blood flow. This is a significant problem in

clinical practice, accounting for up to 10% of early graft failures following liver transplantation, and it predisposes to acute and chronic rejection (Howard 1990, Fellstrom 1998).

During liver resection, it may be necessary to restrict hepatic inflow for a period of time to minimise blood loss, and for technical reasons. The liver remains in situ and therefore suffers “warm ischaemia”, when the cellular metabolism attempts to continue within the limits of the hypoxic environment. After the resection has been performed, the hepatic vessels are unclamped and blood flow recommences, reperfusion of the liver (figure 1.1). During harvesting of the liver for transplantation, the liver is perfused with cold preservative solution, and then removed from the body and stored on ice prior to its transplantation. This subjects the organ to “cold ischaemia”. After the ischaemic period, there is a short period of time when the liver has been replaced in the abdominal cavity whilst vessel continuity is being restored, where it undergoes warm ischaemia. After the hepatic inflow is unclamped the organ is implanted and the liver is reperfused (figure 1.1).

In the above situations, the ischaemia reperfusion injury is a planned surgical insult to the liver and as such, treatments may be developed to help minimise any damage. The development of such therapies requires an understanding of the complex series of cellular events that occur during ischaemia and reperfusion.

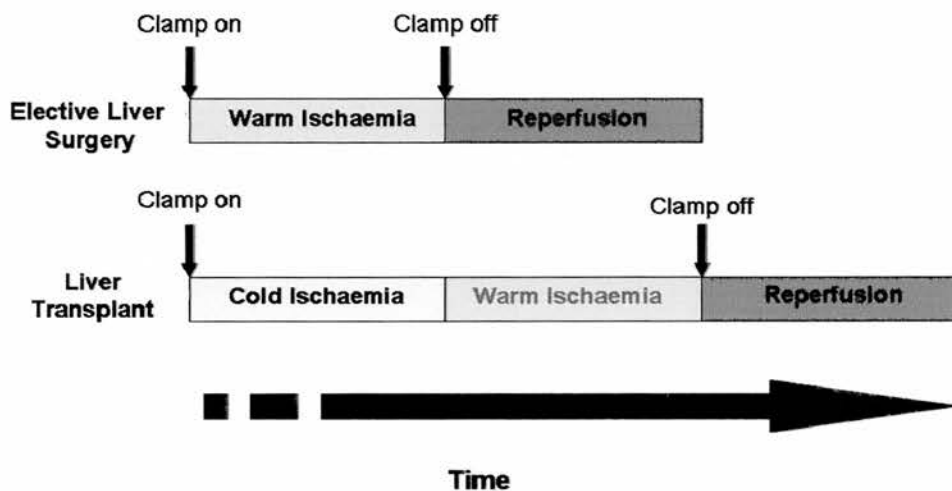


Figure 1.1. Schematic representation of the different insults that occur during liver resectional surgery and liver transplantation.

In elective liver surgery warm ischaemia occurs when the hepatic inflow is clamped. Reperfusion occurs once the surgery has been performed and the clamp is removed. The same process occurs during liver transplantation. However, the organ is cooled at the time of harvest, resulting in a period of cold ischaemia. Once the organ is ready for re-implantation, it is placed within the abdomen whilst the blood vessels are being anastomosed. This leads to a short period of warm ischaemia. Reperfusion occurs once the vessel continuity has been restored and the clamps are removed. See text for full description.

1.2.1 Cellular Events

The various cell types of the liver are differentially affected by ischaemia and reperfusion. Structural changes include cellular swelling, endothelial cell shrinkage, neutrophil infiltration and cell loss. These changes are the result of intracellular events (figure 1.2).

Hypoxia leads to depletion of cellular energy stores (namely ATP), inhibiting homeostatic pump function and resulting in cellular sodium accumulation and swelling (Lemasters 1997). Depending on the duration of ischaemia and degree of ATP depletion, the mitochondrial membrane potential may be lost, which can lead to cell death either by apoptosis or necrosis (Kim 2003). Formation of the mitochondrial permeability transition pore (MPTP) is a critical component of this process in ischaemia-reperfusion injury (Green 2004). Formation of this pore occurs in response to hypoxia and oxidative stress, both of which occur in ischaemia-reperfusion injury (IRI). It requires the assembly of multiple proteins, including cyclophilin D, Bcl_{xl} and adenine nucleotide translocases. The pore forms a junction between the inner and outer membranes of the mitochondria, causing short circuiting of the electron transport chain and cytochrome c release. Apoptosis is triggered by cytochrome c release from the disrupted mitochondria, resulting in caspase activation (Jaeschke 2003a). In hepatic IRI, both apoptosis and cell necrosis occur, dependent on the degree of damage and cellular ATP levels, a process termed necrapoptosis (Jaeschke 2003b).

On reperfusion, endothelial cell injury results in activation of the endothelium, upregulation of adhesion molecules and neutrophil recruitment. This is exacerbated by areas of endothelial cell loss due to apoptosis (Kupiec-Weglinski 2005). These recruited, activated neutrophils generate elastase, matrix metalloproteinases and reactive oxygen species (ROS), resulting in localised tissue damage (Okajima 2004, Sievert 2003). Kupffer cells are activated by ischaemia –reperfusion injury (IRI), triggering production of ROS and proinflammatory cytokines such as $\text{TNF}\alpha$, IL-1 and IFN- γ (Menger 1999). These are damaging to surrounding cells, may trigger apoptosis, and contribute to the activation of neutrophils and the endothelium, aggravating tissue damage. Necrotic debris from dead cells also increases the inflammatory state (Jaeschke 2003).

As a result of proteinase activity during ischaemia, xanthine oxidase is formed from xanthine dehydrogenase. It uses hypoxanthine as a substrate to generate O_2^\cdot and H_2O_2 . Hypoxanthine accumulates during ischaemia as a result of ATP catalysis. This allows rapid generation of significant levels of ROS on reperfusion (Van Bilsen 1989). Endothelial cells and hepatocytes also contain an NADPH oxidase which generates further ROS (Ray 2005). In addition, disruption of the sinusoidal epithelial barrier and changes in the red blood cells results in extravasation of red blood cells and the liberated free heme acts as a further source of ROS, exacerbating the extent of the insult (Teoh 2003).

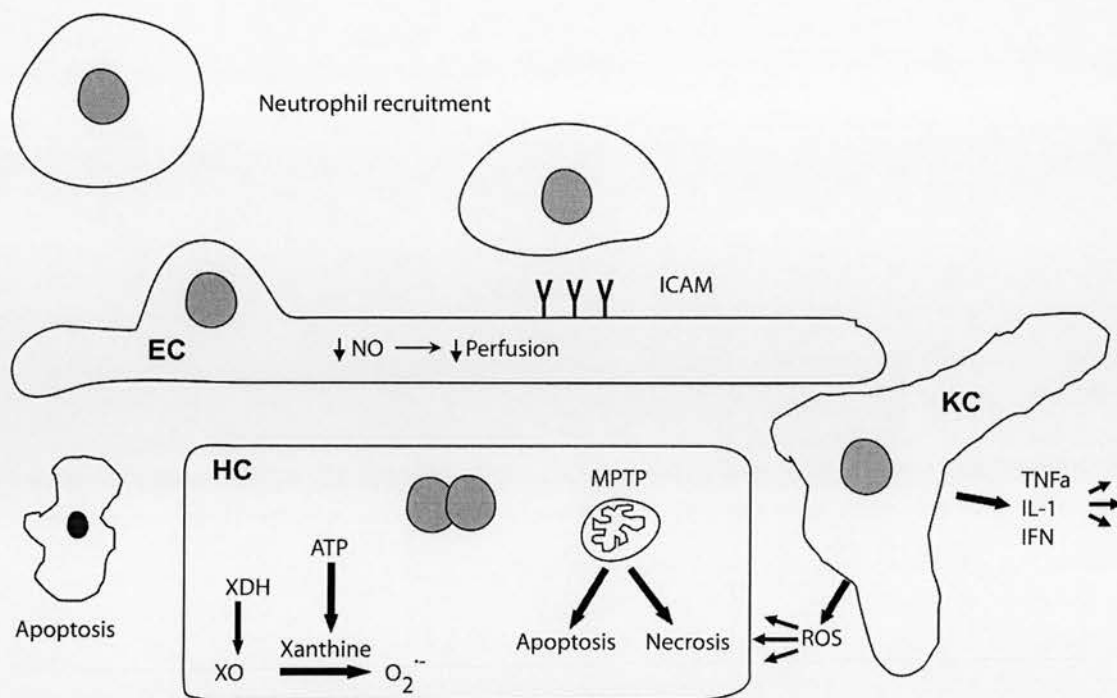


Figure 1.2. Diagrammatic representation of some of the cellular events which occur in the liver during reperfusion after an ischaemic period.

Endothelial cells are activated by the process of reperfusion, leading to expression of cell surface adhesion molecules such as ICAMs. In conjunction with reduced nitric oxide production causing vasospasm, and loss of endothelial continuity, this results in neutrophil recruitment and activation, which then infiltrate the tissue. Some hepatocytes undergo necrosis or apoptosis as an immediate result of the cell damage. During ischaemia, there is formation of xanthine oxidase (see text), and an accumulation of xanthine from ATP catabolism. On reperfusion, XO-mediated degradation of xanthine generates superoxide radicals ($O_2^{\cdot -}$). Activated Kupffer cells also release ROS and inflammatory mediators. **EC** endothelial cell; **HC** hepatocyte; **KC** Kupffer cell; **XO** xanthine oxidase; **XDH** xanthine dehydrogenase; **MPTP** mitochondrial permeability transition pore opening; **ROS** reactive oxygen species.

Nitric oxide (NO) is an important factor in maintaining the microcirculation of the liver. It exerts its effect through activation of soluble guanylyl cyclase, increasing production of cGMP. This intracellular second messenger system acts through cGMP dependent kinases to cause protein phosphorylation, the effects of which include smooth muscle relaxation (Murad 2006). During normal physiological conditions, the vasodilatory effects of NO and the vasoconstrictor endothelin-1 achieve a physiological balance, through which precise control of the hepatic microcirculation is achieved (Paxian 2004). IRI increases the level of endothelin-1 in the circulation and the liver, whilst NO levels are initially low during reperfusion (Kim 2004). This imbalance results in sinusoidal constriction and reduced perfusion of the organ during reperfusion, and the slow flow aids neutrophil recruitment into the damaged organ (Uhlmann 2004).

Potentially, NO can react with ROS, resulting in the formation of nitrogen radicals such as peroxynitrite, which could contribute to cellular damage in hepatic ischaemia-reperfusion injury (IRI). Supporting this, inducible nitric oxide synthase (iNOS) is increased following hepatic IRI, nitrotyrosine is detected in those areas of the liver with histological damage and inhibition of iNOS attenuates hepatic damage after experimental IRI in a rat model (Serracino-Inglott 2003, Takamatsu 2006). However, a different group has demonstrated that exogenous administration of peroxynitrite reduces leukocyte recruitment into the liver following IRI, and inhibition of NOS, whilst reducing peroxynitrite formation, leads to increased neutrophil accumulation and an exacerbation of hepatic injury (Liu 1998, Liu 2000). This apparent contradiction could be the result of the specificity of different NOS

inhibitors used in the two studies as L-NAME (used by Liu) inhibits both endothelial NOS (eNOS) and iNOS, whilst ONO-1714 is believed to be iNOS specific (Takamatsu 2006). Hence it would appear that the production of peroxynitrite from endothelial cells limits neutrophil recruitment and acts to reduce hepatic injury, whilst iNOS-derived peroxynitrite from either resident liver cells or recruited leukocytes causes hepatic damage.

Other cells can contribute to the degree of injury seen in hepatic IRI. There is evidence that the immune response is activated following IRI. This occurs through multiple mechanisms, and involves activation of both the innate and adaptive immune system. The innate immune system appears to be activated through Toll-Like Receptors (TLRs) (Wu 2004). These receptors are involved in the recognition and presentation of microbial antigens, and are present on Kupffer cells, B cells and dendritic cells. TLR activation leads to MAPK and NF κ B activation, which results in antigen specific production of cytokines and chemokines, complement activation and neutrophil recruitment. There is increasing evidence that TLRs are activated following IRI. TLRs are not only activated in response to bacterial components, and endogenous ligands can also trigger activation. In particular, heat shock proteins released from necrotic cells have been implicated in TLR activation (Vabulas 2002). There is increased TLR4 present in Kupffer cells in rat liver transplantation, and TLR4 deficiency reduces the severity of hepatic IRI in mice (Peng 2004, Wu 2004).

T cell activation can also contribute to ischaemia reperfusion injury. It was noted many years ago that systemic immunosuppression with calcineurin inhibitors can

reduce the severity of hepatic ischaemia reperfusion injury, implicating T cell involvement (Suzuki 1993). This has been confirmed in a T cell knockout model of renal IRI, where the severity of injury was reduced in the absence of T cells, and adoptive transfer of T cells restored the severity to that seen in wild-type animals (Ysebaert 2004). The role of the T cell is not entirely clear, but it appears to increase neutrophil recruitment (Zwacka 1997). Furthermore, sinusoidal endothelial cells can present antigens to T cells (Lohse 1996), and T cells are seen to adhere to sinusoidal endothelial cells following cold ischaemia (Clavien 1993).

Ischaemia-reperfusion in the liver is different to that in other organs due to the hepatic anatomy. The liver receives a proportion of its blood supply from the arterial circulation, but in addition also receives de-oxygenated blood from the portal circulation. During hepatic inflow clamping, the portal tract becomes congested, resulting in increased ROS production and translocation of bacteria from the intestine, leading to increased levels of endotoxin. This endotoxic load augments the IRI in the liver and will contribute to TLR activation (Dai 1998).

Circulating factors also play a role in the pathophysiology of hepatic IRI. The complement cascade is activated by the release of cellular debris. C5a increases neutrophil recruitment and aids in activation of Kupffer and endothelial cells, and the membrane attack complex causes direct cell injury (Arumugam 2004). Also, reperfusion of the liver does not occur in isolation. Cytokines and vasoactive mediators released during hepatic reperfusion can cause haemodynamic impairment

and secondary insults to the organ due to underperfusion. This further exacerbates the regional perfusion abnormalities within the liver (Chouker 2004).

1.2.2 Cellular Signalling Mechanisms

Ischaemia-reperfusion activates multiple intracellular signalling pathways, through which some of the above changes are mediated, for example increased expression of pro-inflammatory cytokines such as TNF α and IL-1. These pathways are complex and cannot be seen in isolation, as there is cross-talk between them which helps to integrate their signals. Most of these signals result in the activation or inhibition of transcription factors, and hence alter gene expression. However, some kinases have direct effects on existing aspects of the cell machinery.

Mitogen activated protein kinases (MAP Kinases, MAPKs) are a super-family of serine/threonine kinases which form important signalling pathways transmitting signals from the cell surface to the nucleus (Fang 2005). There are three main families of MAPK: c-Jun N-terminal kinases (JNK), extracellular-signal-regulated kinases (ERK) and p38-MAPK. Each family is organised in functional modules, which are composed of a series of upstream kinases which mediate signal transduction. All three pathways are activated following hepatic ischaemia and reperfusion (Bendinelli 1996, Zwacka 1998, Kobayashi 2002).

JNK activation is a key orchestrator of hepatocyte death from a wide variety of stimuli (Czaja 2003). It contributes to cell death following liver ischaemia-

reperfusion injury (Uehara 2005). The mechanism is not clear, but may result from increased production of TNF α through activation of the transcription factor AP-1 (Becker 1999), or through phosphorylation and activation of the proapoptotic protein Bad (Donovan 2002).

The NF κ B (Nuclear Factor κ B) transcription factor is also activated by hepatic ischaemia-reperfusion (Zwacka 1998). The mammalian NF κ B family contains five subunits (p50, p52, p65, RelA and RelB), and requires a homodimer or heterodimer of two subunits to form a transcriptionally active complex (Ghosh 1998). Their transcriptional effect varies between the different types of dimer (Gerondakis 1999). The prototypical NF κ B dimer (p50/p65) exists as an inactive form in the cytoplasm, complexed with an inhibitor protein called Inhibitor of NF κ B (I κ B), of which there are also several different forms (for example I κ B α and I κ B β) (Luedde 2006). I κ B masks the nuclear localisation sequence and DNA binding sequence of NF κ B, maintaining it in the inactive form. For NF κ B to become activated, I κ B must be serine phosphorylated by I κ B Kinase (IKK) (Karin 1999). Once phosphorylated, I κ B dissociates, is polyubiquitinated and degraded. NF κ B then migrates to the nucleus and binds to the promoter of the target gene.

TNF α release from activated Kupffer cells activates NF κ B through binding to the TNF receptor (TNF-R1) and subsequent activation of IKK (Nakamitsu 2001, Luedde 2006). TNF α has been found to be a critical mediator of apoptosis in hepatic ischaemia-reperfusion (Rudiger 2002). However, following ischaemia-reperfusion injury, NF κ B activation can also occur through tyrosine phosphorylation of I κ B by

the kinase c-Src (Fan 2004). Irrespective of the pathway through which it occurs, NF κ B activation contributes to the liver injury following ischaemia-reperfusion (Fan 2004, Luedde 2005).

1.3 Preconditioning in the Liver

Preconditioning has significant potential for clinical applications in the liver.

Surgical manipulation of the liver, either during resectional surgery or transplantation, result in damage to the organ, which can lead to post operative impairment or failure of the organ, and even death. Any treatment which improves the tolerance of the liver to such insults would have clinical potential. It is for this reason that there is interest in the development of preconditioning strategies for the liver (Selzner 2003).

Broadly speaking, there are two types of preconditioning which have been attempted – physical and pharmacological. Physical techniques include ischaemic preconditioning (Clavien 2003) and heat treatment (Redaelli 2001, Terajima 2000). Pharmacological techniques include the administration of drugs, cytokines and gene transfer techniques (Camargo 1997, He 2006).

1.3.1 Ischaemic Preconditioning

The phenomenon of preconditioning was first identified in the setting of cardiac ischaemia (Murray 1986). Ischaemic preconditioning has since been tried and found to be protective in a wide variety of models, including liver injury (Pang 1995, Heurteaux 1995, Compagnon 2005). During ischaemic preconditioning, the liver is deprived of oxygenated blood for a short period of time, typically 5 to 10 minutes, blood flow is restored for a further period of time, again typically 5 to 10 minutes, and then the surgical procedure or injurious process is commenced. Koti recently reviewed the literature on ischaemic preconditioning in the liver, and the technique has been found to be beneficial in models of total or partial hepatic ischaemia, and to be effective in mice, rats and pigs. It can also protect the liver from both warm and cold ischaemic injury. Ischaemic preconditioning results in reduced liver cell damage, improved ATP levels, lower cytokine expression and reduced neutrophil recruitment following ischaemia reperfusion injury (Koti 2003).

Ischaemic preconditioning (IP) studies have been performed in humans, both in patients undergoing liver resection, and in patients having liver transplantation (Clavien 2003, Azoulay 2005). Clavien's randomised controlled trial in 100 patients undergoing hepatic resection found that IP was of greatest benefit in younger patients, and in those with fatty infiltration of the liver (Clavien 2003). This study demonstrated the proof of principle in humans. However, a trial in human hepatic transplantation demonstrated that although ischaemic preconditioning improved the tolerance of the graft to preservation and reperfusion, it resulted in impaired early

function (Azoulay 2005). Thus, further study with larger numbers is required to understand more fully how to select patients for this intervention, and who is likely to benefit the most.

Ischaemic preconditioning provides two “windows” of protection: the first window is effective immediately and lasts for several hours; the second window is only effective after at least 12 hours and lasts for up to 72 hours (Pagliaro 2001). The different timing of these two windows reflects the different mechanisms through which they work. The first window is thought to be due to rapid changes in the tissues such as: activation of kinase signalling pathways including MAP kinases; generation of adenosine and nitric oxide; and mitochondrial changes. In contrast the second window appears with the production of protective proteins which require time to be synthesised by the cells. These proteins include heat shock proteins and heme oxygenase 1.

1.3.1.1 First window of protection

Mitogen activated protein kinases are activated following hepatic ischaemia and reperfusion (see section 1.2.2). However, ischaemic preconditioning does alter the responsiveness of these pathways to IRI such as inhibiting JNK activation (Izuishi 2006). This change renders hepatocytes resistant to apoptosis (Marderstein 2003) and may contribute to the first window of protection.

NO also plays a role in hepatic preconditioning which appears to be mediated through stimulation of p38 MAPK via cGMP dependent pathways, rather than through modulation of PKC (Carini 2003). This may be an effect of adenosine release, which could stimulate nitric oxide synthase in endothelial cells (Serracino-Inglott 2002).

Adenosine generated from ADP breakdown interacts with cell surface receptors, and appears to be central to ischaemic preconditioning (IP), with exogenous delivery of adenosine being able to mimic IP (Peralta 1997). Activation of the A₁ or A₃ receptor is important in myocardial preconditioning (De Jonge 2002). However, in the liver, preconditioning appears to be mediated through A₂ receptors, as shown by inhibitor studies (Peralta 1999, Nakayama 1999). Delineation of this pathway has shown that adenosine receptors signal through PI3K or phospholipase C, activating PKC δ and ϵ , the target of which is stimulation of p38 MAPK (Carini 2004). PKC activity has been shown to be essential for hepatic IP in a porcine cold ischaemia model (Ricciardi 2001).

Thus it appears that the first window of ischaemic preconditioning is effected through, activation of p38 MAPK, which results in increased tolerance of the cells to injury, and initiates cellular proliferation which is required as regenerative response. The mechanism through which p38 works is unclear at present, although downstream factors which may be involved include MAPKAPK2, and the transcription factors ATF-2 and MEF2C (Nakano 2000).

Improved preservation of cellular homeostasis also contributes, and IP improves the maintenance of intracellular pH which may prevent ATP depletion (Carini 2000, Peralta 2000). IP also reduces production of xanthine and xanthine oxidase, thus reducing oxidative stress on reperfusion (Fernandez 2002).

1.3.1.2 Second window of protection

The second window of preconditioning is characterised by adaptations in the liver which generally require the synthesis of new proteins, and it lasts longer than the first window, being effective from approximately 12 hours up to 72 hours depending on the model used. Many of the newly synthesised proteins are heat shock proteins, including Hsp70 and heme oxygenase 1. Some researchers believe that these are only markers of the second window, and that they do not provide the protective effect seen (Pagliaro 2001), although this is debated. Hsps are numerous in class and function, but generally help to maintain cellular homeostasis. Other synthesised proteins include cyclin D, iNOS and MnSOD, which stimulate cell proliferation and increase the defence against antioxidants (Eisen 2004).

1.3.2 Other Physical Preconditioning Methods

Remote preconditioning is essentially a modification of ischaemic preconditioning, whereby a site distant from that undergoing the injury, such as a limb, is subjected to the preconditioning stimulus. This appears to have similar efficacy to that of ischaemic preconditioning within the target organ itself (Kharbanda 2002). It may rely on similar mechanisms to “normal” ischaemic preconditioning, and the release of humoral factors is thought to play a significant role, although this has not yet been fully elucidated (Dickson 1999).

Heat preconditioning involves subjecting the target organ to a period of elevated temperature (“heat shock”), leading to the synthesis of proteins which protect the tissue against further injury, and this has been shown to be protective in the liver in both ischaemia-reperfusion injury and transplant-associated injury (Terajima 2000, Mokuno 2004). These newly synthesised proteins are called heat shock proteins and have several specialised functions. Many have chaperone properties and help to maintain the functional integrity of cellular machinery. Of note, heat shock can also inhibit NF κ B, and this may account for some of its effects, in addition to the effect of the heat shock protein production (DeMeester 2001, Chen 2005).

Whilst heat preconditioning has been used in animal and cell models, there are practical difficulties in subjecting a human subject to whole body hyperthermia. Induction of heat shock proteins can be achieved by individuals spending time in a sauna or through incremental exercise (Currie 2004). However, it is likely to be

unfeasible in routine clinical practice. Nonetheless a biological understanding of how these proteins are induced and how they exert their cellular effects is of interest and may aid the development of specific pharmacological agents which can induce these proteins or target their processes.

1.3.3 Pharmacological Preconditioning

Pharmacological preconditioning encompasses a variety of techniques. This ranges from commercially available drugs previously used in different fields, to genetic techniques such as gene transfer. The agents used vary in their specificity and in their functional target (Selzner 2003). As described in section 1.2, the pathophysiology of ischaemia reperfusion injury is complex and multifactorial, thus providing numerous possible avenues to explore for the development of therapeutic targets. Many of the approaches attempted for pharmacological preconditioning have been addressed at specific events that occur during hepatic IRI, whilst others have been identified from studies on ischaemic preconditioning. All methods involve either inhibiting a deleterious cellular process or upregulating a beneficial cell survival factor or pathway. This is often achieved through induction of a degree of cellular stress, which results in activation of the stress response (see section 1.4).

Ischaemia reperfusion injury is characterised by production of reactive oxygen species from multiple cell types (see section 1.2.1), and several groups have used anti-oxidant approaches to achieve preconditioning. Overexpression of superoxide dismutase (which degrades superoxide radicals) reduces the severity of hepatic IRI

(Zwacka 1998, Yabe 2001, Wheeler 2001). Reduction of hepatic IRI has also been found with allopurinol, which inhibits xanthine oxidase (Karwinski 1997), and catalase which degrades hydrogen peroxide (Tanaka 1990).

Mitochondrial dysfunction and disruption releases cytochrome C and leads to caspase activation and apoptosis following hepatic IRI (Lemasters 1998, Cohen 1997). Caspase inhibitors reduce the severity of hepatic IRI however they do not completely prevent it, suggesting that while they inhibit apoptosis, they have no effect on necrosis (Cursio 1999 & 2000). Cyclosporin prevents the mitochondrial permeability transition (Pastorino 1993) and also reduces liver IRI (Kurokawa 1992).

In addition to caspases, other degradative enzymes are activated by hepatic IRI, including calpains and matrix metalloproteinases. Calpains are calcium dependent cysteine proteases which degrade membrane proteins, and contribute to apoptosis and necrosis and hepatic IRI (Squier 1994, Arora 1996, Kohli 1997), and their inhibition reduces the degree of hepatocyte and endothelial cell death (Kohli 1997, Sindram 1999). Methylprednisolone has been known for many years to protect the liver from IRI (Fornander 1984), and it can be as effective as IP (Glanemann 2004). Prednisolone reduces calpain activation, and this may contribute to its effects (Wang 2001).

Matrix metalloproteinases (MMPs) are important in the degradation and turnover of extracellular matrix. They are induced following ischaemia reperfusion in the liver, and the inhibitor RXPO3 reduces hepatic damage following IRI (Cursio 2002). The

inhibition of MMPs may contribute to the preservative effects of University of Wisconsin solution, which is currently the standard preservative for cold preservation of human liver transplants (Upadhy 2000).

Hepatic nitric oxide levels are low following reperfusion (Kim 2004), and this may contribute to regional perfusion abnormalities. Adenosine has preconditioning properties, and may work through increasing nitric oxide production. It increases nitric oxide production in the ischaemic liver (Peralta 1999), which prevents damage to hepatocytes and endothelial cells (Cottart 1999). Adenosine was initially identified as an important mediator of ischaemic preconditioning in the heart (Heidland 2000). Whilst the A1 receptor is important in cardiac preconditioning, the A2A receptor appears to be central to hepatic preconditioning. A selective agonist of the A2A receptor exists – ATL146e. In a mouse model of hepatic ischaemia, it reduces hepatocyte necrosis and leucocyte infiltration (Day 2004). Importantly, treatment with ATL146e is still effective when administered at the time of reperfusion, of even up to 1 hour after, presenting an attractive profile for the drug in clinical use. In the above study, ATL146e reduced the level of pro-inflammatory cytokines produced following IRI, including IL-1 α , IL-6, RANTES and IFN- γ (Day 2004), which may also contribute to its effects.

Nitric oxide itself exerts many of its effects through activation of soluble guanylyl cyclase and increased production of cGMP. Through its effect on smooth muscle relaxation and inhibition of platelets (Murad 2006), increasing cGMP may ameliorate regional perfusion abnormalities in hepatic IRI, and nitric oxide donors induce

protection against hepatic IRI (Dhar 1998, Cottart 1999). Infusion of atrial natriuretic peptide (ANP) reproduces the effects of IP, reducing hepatic IRI and improving graft survival after liver transplantation in a cyclic GMP (cGMP) dependent process (Bilzer 1994 and 1999).

As described above, there are innumerable strategies through which to attempt pharmacological preconditioning. Many of the pharmacological agents described above have no established safety profile in humans, and have limited applicability to clinical practice. They may also have unforeseen adverse effects, either through non-specific inhibition of a vital system, or through specific inhibition of the targeted process which impairs the function of other organs. This is the main reason that few of these pharmacological strategies have yet made the transition to clinical trials. Matrix metalloproteinase inhibitors have been trialled in chronic diseases, but have had significant side effects (Brown 2000).

This thesis examines the potential of two agents, which are suitable for administration to patients, to induce a preconditioned phenotype in human hepatocytes, through harnessing of the innate stress response, which will be described in the following sections.

1.4 The Stress Response

The stress response is a broad term which encompasses all of the ways in which a cell or target organ responds to stress of any nature (Welch 1993). It incorporates the upregulation of genes and alteration of cellular mechanisms in response to an injurious stimulus and is exemplified by the induction of particular protective proteins which prevent or lessen the severity of damage sustained. It was first described in response to elevated temperature (Ritossa 1962), and hence has subsequently been known as the heat shock response. A large number of stress-inducible proteins have been identified, and many have been termed heat shock proteins due to the stimulus with which they were first studied.

1.4.1 The Heat Shock Response

The heat shock response can be stimulated by a brief elevation of temperature to approximately 5°C above normal. The significance of this response is that the tissue is then rendered tolerant to subsequent heat injury. Detailed research has shown that this response also occurs following many diverse stimuli including mechanical stress, oxidants, cytokines and infection (Benjamin 1998). The protection afforded by this response extends to other noxious stimuli, including hypoxia and oxidative stress. For this reason the stress response is the term currently favoured.

1.4.2 Classification of heat shock proteins

Many of the proteins involved in the stress response have been called heat shock proteins (Hsps) as a virtue of the context in which they were first studied. Others are termed glucose regulated proteins (Grps) as they were first identified in response to periods of glucose deprivation. These proteins are grouped into families on the basis of their molecular weight. Certain heat shock proteins are constitutively expressed, whilst others are only inducible in response to a stimulus. Thus, in addition to being described according to their molecular weight and method of induction (e.g. Hsp70), there are inducible and constitutive forms i.e. Hsp70i and Hsp70c.

1.4.3 Functions of Heat Shock Proteins

There is great diversity of heat shock proteins which have specialised roles. Some are limited to specific intracellular compartments and have highly specialised functions, whilst others are more ubiquitous. Most heat shock proteins function as molecular chaperones. Molecular chaperones mediate the correct folding of other proteins, but do not take part in their final assembly (Fink 1999). Protein mis-folding is a common result of cellular injury. It results in exposure of hydrophobic parts of proteins which would have been hidden when the protein was in its native state. These hydrophobic regions stick to other proteins, causing aggregations of proteins to form, which can interfere with normal cellular machinery. Chaperones work by associating with these damaged proteins, shielding them from the rest of the cellular environment. The chaperone functions are highly specialised, and specific chaperones function to

safely transport the damaged protein around the cell (e.g. Hsp70), whilst others work to allow refolding of the protein to occur (e.g. Hsp60) (Fink 1999).

Many heat shock proteins have functions other than simple chaperones. Hsps can display cytokine-like activity. Soluble Hsps released from cells can bind to the cell surface Toll-like receptor TLR4/CD14 or CD91 (Kol 2000, Tobian 2004), and trigger intracellular signalling through MAP kinase pathways (Kol 2000, Sasu 2001).

Hsps can also play a role in antigen presentation. They are known to associate with cellular peptides, including antigenic peptides. Lysates from tumour cells are rich in hsps, which may be associated with tumour antigens, and vaccination with the hsp fraction from tumours has had very promising results in improving the survival after many cancers, including colon cancer (Mazzaferro 2003). This may be a result of their ability to induce maturation of dendritic cells (Somersan 2001)

1.4.4 Heat Shock Protein 70

Hsp70 is a ubiquitous Hsp, which exists in a constitutive form (Hsp70c, also termed Hsc70) and an inducible form (Hsp70i). Hsp70c is expressed in all tissues, with very low or non-existent basal levels of Hsp70i in non-stressed cells. Following stress, Hsp70i is rapidly upregulated. Hsp70 can bind to unfolded or nascent proteins, and then either refold the protein itself, transport it safely around the cell and deliver it to other machinery that can refold the protein, or target it for destruction by delivering it to the proteosome.

In resting cells, Hsp70 stabilises unfolded nascent peptides and delivers them to their destined intracellular compartment (Beckmann 1990, Artigues 1998). It also targets misfolded proteins to lysosomes (Agarraberes 1997). Hsp70 may also have a role in the maintenance of cellular structure, as it binds to actin and tubulin (Liang 1997).

Hsp70 has two important functional domains – a protein binding domain and an ATPase domain. The conversion of ATP to ADP allows the release of the newly folded protein. Thus binding is more stable in periods of low ATP levels, such as occurs during stress (Mayer 2005).

Overexpression of Hsp70 can reduce lipid peroxidation by hydrogen peroxide (Su 1999), and it can protect against TNF α associated lethality (Van Molle 2002). In vitro it protects human hepatocytes from oxidative stress (Hosoi 2002). In vivo, it protects the liver from both chemical and ischaemia-reperfusion injury (Lee 2004, Boeri 2003).

1.4.5 Other Heat Shock Proteins

Hsp90 forms an essential part of the steroid receptor. It can also bind to kinases, microtubules and intermediate filaments. Hsp90 has been found to be associated with inactive HSF-1 and it may contribute to the regulation of HSF-1 activation (Duina 1998).

Hsp60 (also known as chaperonin) forms a large hetero-oligomeric complex, in association with Hsp10, which functions to refold misfolded protein. Stacks of Hsp60 units form an enclosed space to which mis-folded protein is delivered. Hsp10 functions as a lid on the barrel. Within this space, the dynamics favour refolding of the protein. Hsp10 then dissociates and the refolded protein is released (Fink 1999).

The “small Hsp” group includes Hsp27 (Hsp27 in humans, equivalent of Hsp25 in rodents) and $\alpha\beta$ crystallin. These form large oligomeric complexes and can bind a wide variety of substrates (Stromer 2003). In contrast to Hsp60, unfolded proteins bind to the surface of the oligomeric sHsp complex, and remain accessible to other members of the Hsp family. It has been suggested that these complexes act as a sump for unfolded proteins during times of cellular stress. After removal of the stress, the unfolded proteins are available for binding to Hsp70, which can then perform its function to refold the proteins, or to transport them to other areas of the cell (Dillmann 1999).

1.4.6 Heat Shock Factor 1

Heat shock factor 1 (HSF-1) is the predominant transcription factor responsible for the induction of Hsps in response to heat. Other HSFs exist (HSF 1-4 respectively). HSF 2 appears to be important in development and differentiation. Although it is not responsive to stress, it may potentiate HSF-1 activity in response to heat shock (He 2003). HSF-3 has only been identified in avian cells, and HSF-4 has not yet been fully characterised (Pirkkala 2001). HSF-1 can be activated by mechanisms other

than heat. These include, oxidising agents and heavy metals (Zou 1998), osmotic stress (Caruccio 1997), and compounds such as salicylate and indomethacin (Cotto 1996).

The structure of HSF-1 is complex, comprising of a trimerisation domain, a regulatory domain and an activation domain. Each of these domains function as their name suggests, and it is the regulatory domain that is responsible for the heat sensing ability of HSF-1 (Newton 1996).

HSF-1 exists as an inactive monomer in the cytoplasm of resting cells, and on exposure to heat, multiple events occur to activate it. It becomes hyperphosphorylated, undergoes trimerisation and migrates to the nucleus. Once there it binds to DNA and upregulates target genes by binding to heat shock elements (HSEs) in their promoter. This contains the motif 5'-nGAAn-3' (Sarge 1993, Zuo 1995). Following activation, HSF-1 remains in the nucleus in "stress granules" bound to heterochromatin. These gradually disappear during the recovery phase (Cotto 1997).

The process of HSF-1 activation is not fully understood. It is subject to many activating and repressing factors (figure 1.3). Phosphorylation appears to play a key role in both activation and repression of HSF-1.

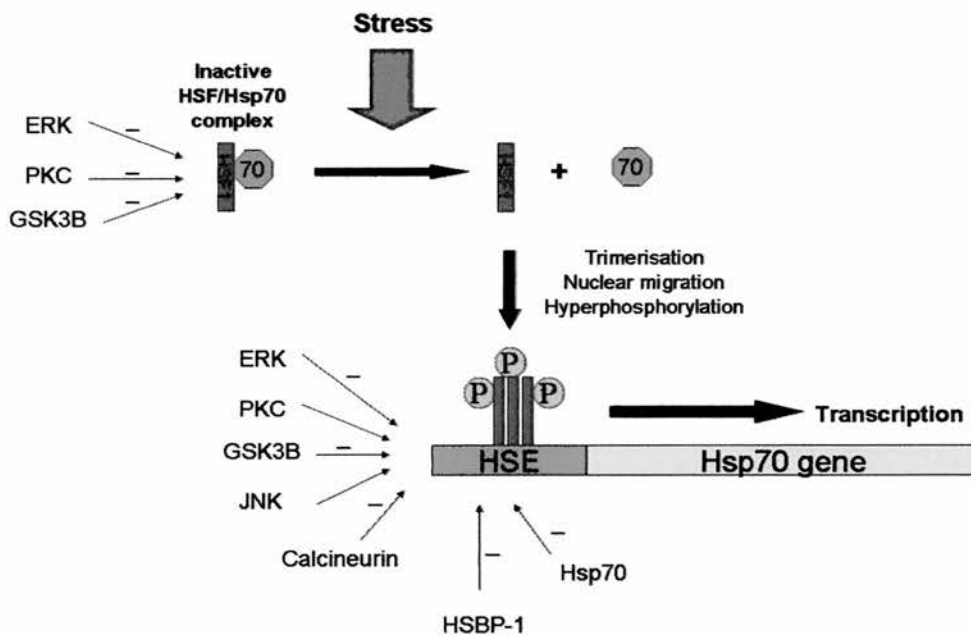


Figure 1.3. Diagrammatic representation of HSF-1 activation and the multiple inhibitory pathways which repress HSF-1 activity.

HSF-1 is usually present in an inactive state in the cytoplasm of resting cells, in association with either Hsp70 or Hsp90 which inhibits HSF-1 activation. Further inhibitory pressures are exerted by the activity of several kinases that phosphorylate inhibitory residues of HSF-1. The presence of a stress such as heat shock leads to dissociation of Hsp70, and a series of changes in HSF-1 which lead to it migrating to the nucleus, binding to its consensus sequence (the heat shock element – **HSE**) and gaining transcriptional activity. Further inhibitory kinases act to limit the duration of HSF-1 activity and to facilitate its inactivation upon the removal of the stressful stimulus.

HSF-1 in resting cells is phosphorylated on specific residues, which results in repression of transcriptional activity. This is mediated through phosphorylation of the regulatory domain, the region from amino acid residues 201 to 370 (Newton 1996). This contains several serine residues which are targets for MAP kinases. Serine 307 is phosphorylated by ERK, which subsequently allows serine 303 to be phosphorylated by GSK-3 (Chu 1996, Kim 1997). Also, PKC α and ζ can phosphorylate serine 363 (Chu 1998). These three sites are responsible for repression of the heat shock response at normal temperatures. Furthermore, the 303/307 phosphorylation allows binding of HSF-1 to the signalling molecule 14-3-3 epsilon, which is involved in the nuclear export and cytoplasmic sequestration of HSF-1, thus providing further repression of transcriptional activity (Wang 2003).

Inducible phosphorylation of HSF-1 is essential for it to acquire transcriptional activity (Cotto 1996, Ding 1997). The specific kinases responsible have proven to be elusive, although at least four serine/threonine residues are inducibly phosphorylated in response to heat shock (Xia 1997). Protein kinase CK2 is activated by heat, and can phosphorylate HSF-1 (on threonine residue 142). Mutation analysis has suggested that this phosphorylation is essential for the activation of HSF-1, although this is unlikely to be the sole regulatory step, as overexpression of CK2 does not result in the same degree of transactivation as that seen with heat shock (Soncin 2003). Phosphorylation of serine 230, possibly by CAMK II, also contributes to the magnitude of the HSF-1 transcriptional activity (Holmberg 2001). Thus it appears that many different pathways contribute to activation of HSF-1, providing the capability for fine regulation of the degree of response to a given stimulus.

Other changes in the phosphorylation of HSF-1 are important in the cessation or down regulation of its activity after its initial activation. ERK-1, GSK-3 and JNK are all activated by heat shock, and may contribute to the limitation of HSF-1 activity by phosphorylation of the sites detailed above (Kim 1997, Dai 2000).

Dephosphorylation of HSF-1 is likely to be an important method of switching off HSF-1, and phosphatase inhibition prevents dissociation of HSF-1 trimers (Xia 1997). This has not been completely elucidated, but the calcium and calmodulin dependent protein phosphatase calcineurin has been implicated in this regard (Soncin 2000).

Other proteins also contribute to the repression of HSF-1 activation. One such negative regulator is the Heat Shock Factor Binding Protein 1 (HSBP1). This nuclear protein only associates with active, hyperphosphorylated HSF-1. It associates with the trimerisation motif and reduces its transcriptional activity (Satyal 1998). Hsp70 also interacts with activated HSF-1 (Baler 1992), and associates with the activation domain. This represses transcriptional activity and represents an autoregulatory step where the heat shock response limits itself by the action of the nascent Hsp70 (Shi 1998). Interestingly, HSBP-1 also interacts with Hsp70. The HSBP-1/HSF-1 interaction occurs prior to the Hsp70/HSF-1 interaction and these two events temporally coincide with the attenuation of transcriptional activity and the disassembly of the HSF-1 trimer respectively, suggesting a possible multistep attenuation process (Satyal 1998).

The promoter regions of stress responsive genes commonly contain motifs for many transcription factors including HSF-1, and these can potentially integrate multiple signals regulating expression of the target gene. In the human Hsp70 and Hsp90 promoters, there is a combined HSE and STAT binding site, and study has shown that not only can HSF-1 and STAT-1 have an additive effect on transactivation of the gene, but that they also physically interact with each other (Stephanou 1999). Indeed, both HSF-1 and STAT-1 seem to be required for the production of Hsp27 in response to heat (Yokota 2003). In contrast, while both STAT-3 and HSF-1 are able to independently activate the Hsp90 promoter, they are mutually antagonistic, and activation of the gene is reduced in the presence of both factors (Stephanou 1998). Thus regulation of HSF-1 transcriptional activity reflects not only the balance of factors regulating specific changes in HSF-1, but also depends on the influence of other transcription factors and other cellular events.

1.5 Heme Oxygenase 1

Heme oxygenase 1 (HO-1), also termed Hsp32, is a stress protein which does not function as a chaperone. It is an enzyme, which catalyses the degradation of heme proteins, and is the rate limiting step in heme breakdown (Tenhunen 1968). This reaction produces carbon monoxide, biliverdin, and free divalent iron, by cleaving the heme ring at the α -methene bridge (Tenhunen 1968). It is these products which mediate the effects of HO-1 (Otterbein 2000). Three different heme oxygenases have been identified – HO-1, HO-2 and HO-3. HO-1 is induced by stress. HO-2 is expressed constitutively, and is located primarily in the brain and testes (Sun 1990, Ewing 1995). HO-3 has a high degree of homology to HO-2, but it lacks significant catalytic activity, and its role is unclear (McCoubrey 1997). Whilst all stress proteins appear to have a protective function, HO-1 appears to be critical for cellular protection from injury (Poss 1997), and it has been shown to provide protection in a variety of conditions. HO-1 expression is induced by different factors in different organisms. Heme rings are the prototypical inducer (Tenhunen 1969), although other classic inducers include heavy metals such as cadmium (Alam 1989).

1.5.1 Mechanism of HO-1 mediated protection

The end products of heme oxygenase activity are biliverdin, carbon monoxide and iron. Each of these products has been shown to contribute to the protective effects of HO-1. The essential nature of enzymatic heme oxygenase activity to provide the

protective effects of upregulated HO-1 has been shown by the loss of protection in times of reduced substrate (Foresti 2001), reinforcing the finding that it is the products of HO activity which produce its effects.

1.5.1.1 Carbon monoxide

Carbon monoxide (CO) has traditionally been viewed as a toxin, and on a large scale this is certainly true. However, in recent years, a role for CO as a signalling molecule similar to nitric oxide (NO) has been established (Otterbein 2003). Evidence that CO provides an important contribution to the observed effects of HO-1 is seen by the reproducibility of many of HO-1's effects by the administration of exogenous CO (Akamatsu 2004, Chauveau 2002). CO exerts its effects through intracellular signalling pathways, including MAP kinases and guanylyl cyclase (Brouard 2000). In particular, its anti-apoptotic effects on endothelial cells appear to be mediated through p38 MAPK (Brouard 2000). This has been further refined, and Zhang has shown that CO activates the p38 α isoform through MKK3 stimulation. This results in a reduction in Fas and Fas-ligand expression, and protection from apoptosis by inhibiting caspase activation and increasing expression of the anti-apoptotic Bcl proteins Bcl-2 and Bcl-xl (Zhang 2003).

Carbon monoxide has the potential to bind to heme proteins and other heavy metal containing proteins. As such, it is capable of binding to cyclo-oxygenase, cytochrome p450 reductase, and iNOS (Botros 2002). It can also bind to transcription factors with a heme domain, such as Bach1, which is important in the

negative regulation of HO-1, and may represent one part of a complex system of autoregulation.

CO reduces levels of LPS-induced proinflammatory cytokines such as TNF α and IL-1 β , whilst also increasing levels of anti-inflammatory cytokines such as IL-10. This appears to be mediated through MAP Kinase pathways (Otterbein 2000). Hence CO may be responsible for some of the altered cytokine profiles seen with HO-1 induction.

1.5.1.2 Biliverdin

Biliverdin generated from HO-1 activity is rapidly converted into bilirubin by the enzyme biliverdin reductase, and it is helpful to think of biliverdin and bilirubin together when considering their contribution to the effects of HO-1. Both have strong anti-oxidant effects. Bilirubin's anti-oxidant effects are sufficiently potent to be protective against oxidative injury at the same level that it is produced *in vitro* (Clark 2000). That each of the end-products of HO-1 activity contributes to its beneficial effects is reinforced by the finding that in certain models, biliverdin can provide an effect analogous to that seen with elevated HO-1, whilst administration of CO cannot. This has been demonstrated in an inflammatory bowel disease model and in alteration of endothelial cell activation (Otterbein 2003, Soares 2004). This provides direct evidence that although some of the effects may overlap, each of the products of HO-1 have independent contributions to make to the overall effect of HO-1 activity.

1.5.1.3 Iron

Free ferrous iron is generated from the centre of the heme ring by HO-1 activity. This is potentially pro-oxidant, and may take part in intracellular Fenton reactions to generate free radicals (Ligeret 2004). However, the cell responds by rapidly upregulating the iron sequestering protein ferritin, as well as generating increased levels of an ATPase pump which actively exports iron (Eisenstein 1991, Ferris 1999). This therefore limits the detrimental effect of free Fe^{2+} . An augmented exportation of Fe^{2+} may contribute to the cytoprotective effects of HO-1, as the intracellular iron level is critical in contributing to cell death in the face of several stressful stimuli, and the lower levels of intracellular iron seen with HO-1 induction are associated with cell survival (Ferris 1999).

The elevated levels of ferritin also play a role in cellular protection, and exogenously administered ferritin can replicate the effects of HO-1 in some but not all models. Ferritin is responsible for the protection afforded by HO-1 in a hyperoxic lung injury model (Taylor 1998), although protection was independent of ferritin in a model of endotoxic shock (Otterbein 1997).

1.5.2 Transcriptional Regulation of HO-1

The human HO-1 gene is located on chromosome 22. It contains 5 exons, spanning approximately 14kb. Regulation of HO-1 transcription is tightly regulated which can be cell and species specific. The promoter region contains consensus binding sites for

multiple transcription factors, which have both positive and negative effects on transcription, thus allowing fine control of HO-1 expression and a rapid response to a wide variety of stimuli.

Alam has identified two promoter regions which confer responsiveness to most inducers in the mouse HO-1 gene (Alam 1994 and 1995). These regions are termed E1 and E2 (enhancer 1 and 2 respectively; previously termed SX2 and AB1). The inducers which are active include heme, NO, heavy metals, hyperoxia and LPS (Ryter 2002, Poole 2005). It has been proposed that all of these stimuli share a common signal transduction mechanism, centred around a “stress response element” (StRE) in these regions. The StRE comprises of three repeats of the sequence (T/C)GCTGAGTCA. This resembles strongly the AP-1 consensus sequence, and also permits binding of NF-E2 and Nrf2 (Alam 2003).

Agarwal has examined the human HO-1 promoter for these sites, and found similar elements at -4.0kb and -10.6kb respectively, (Sikorski 2004, Hill-Kapturczak 2003). However, these two regions alone do not provide full induction of HO-1 in response to cadmium or heme, and they do not respond to other stimuli such as oxidised lipids, hydrogen peroxide and hyperoxia. An enhancer has, however, been identified which lies within the HO-1 gene itself, and restores the full responsiveness to heme and cadmium (Hill-Kapturczak 2003).

The human HO-1 promoter also contains other interesting regions. The region -1976 to -1655 contains regulatory sites for AP-1, HNF-1, HNF-4, c-Rel and GATA-X,

however, it is functional in the HepG2 hepatoma cell line, but not in HeLa cells, demonstrating that the regulation of HO-1 is likely to be different not only across species, but also between different tissues (Takahashi 1999).

Near to the HO-1 start codon, there is a negative regulatory sequence comprising a variable number of GT repeats, which inhibits transcription if there are many repeats and contributes to inter-individual differences in HO-1 inducibility (Exner 2004). This area also includes a heat shock element (HSE), which has however, been found to be at least functionally silent, or it may even have a repressive action on HO-1 gene transcription (Okinaga 1996, Chou 2005).

1.5.2.1 Nrf2

Nrf2 is fully known as NF-E2 related factor 2. It is a cap'n'collar transcription factor, with a basic leucine zipper (bZIP) region. It is the basic portion which allows DNA binding. The cap'n'collar region is common across all Nrf proteins, but its function is unknown (Jaiswal 2004).

Nrf2 is important in the response to oxidative stress. First demonstrated in the gene NQO1 (NAD(P)H:Quinone Oxidoreductase 1), overexpression of Nrf2 was found to upregulate the induction of NQO1 in response to antioxidants and xenobiotics (Venugopal 1996). Nrf2 has since been found to be an important influence on the expression of several defensive genes including GST Ya, γ -GCS and HO-1 (Wild

1999, Alam 1999, Nguyen 2000). Thus it is a key regulator of the cell's response to oxidative stress.

Nrf2 has several layers of control regulating its activity. It functions by heterodimerising with other bZIP proteins, namely Jun and Maf proteins including c-Jun, MafG, MafK and MafF. This binding is essential for Nrf2 activity. However, small Maf proteins are capable of homodimerising and negatively regulating the expression of Nrf2 dependent genes (Jaiswal 2004). The relationship between which protein dimerises with Nrf2 and the functional effect on transcription is unclear at present (figure 1.4).

Nrf2 also has several negative regulators. Keap1 (also termed INrf2 – inhibitor of Nrf2) acts to retain Nrf2 within the cytoplasm, possibly by binding to actin (Kang 2004). Keap1 may also facilitate the ubiquitination of Nrf2 (McMahon 2003). Keap1 contains a large number of cysteine residues which are responsible for its repression of Nrf2. Mutational analysis of residues C273 and C288 has revealed that these contribute not only to maintaining Nrf2 in the cytoplasm, but also promote its ubiquitinylation (Zhang 2003, Levonen 2004), and this effect will be lost after dissociation of Nrf2 from Keap1, increasing cellular levels. The cysteine residue C151 has also been shown to play a crucial role in the dissociation of Nrf2 from Keap1 following an oxidant stimulus (Levonon 2004), allowing it to migrate to the nucleus. The mechanism through which this occurs is unclear, but may be the result of a post-translational modification of Keap1, involving this residue (Nguyen 2004). It is known that protein kinase C (PKC) phosphorylates Nrf2 which allows its

dissociation from Keap1 (Bloom 2003). Phosphatidylinositol 3-kinase (PI3K) may also play a role in allowing nuclear localisation of Nrf2, although this appears to be mediated through depolymerisation of actin, and may not lead to dissociation from Keap1 (Kang 2002).

Bach1 also represses Nrf2 activity. This is another bZIP protein which interacts with small Maf proteins (Oyake 1996). It heterodimerises with MafK and binds to the StRE, repressing gene expression, apparently by preventing Nrf2 binding (Sun 2002). Cadmium, a potent HO-1 inducer, binds to certain residues of Bach1, and causes its displacement from the StRE, de-repressing gene expression and increasing HO-1 levels (Suzuki 2003). This is possibly the mechanism through which heme upregulates HO-1 expression, although recent data suggests that it may not be the only mechanism (Shan 2004).

Further regulation of Nrf2 is achieved by its short half-life of 13 minutes. It is degraded through the ubiquitin-proteasome pathway, and activation of Nrf2 may be achieved by Nrf2 stabilisation and inhibition of its degradation (Stewart 2003).

Nrf2 activation can be stimulated through a variety of molecular pathways. Carnosol increases HO-1 through Nrf2 activation, and this is associated with activation of the ERK, p38 and PI3K systems, of which PI3K and its downstream kinase AKT appear to be instrumental (Martin 2004). Diallyl sulfide activation of Nrf2 requires ERK and p38 activity (Gong 2004).

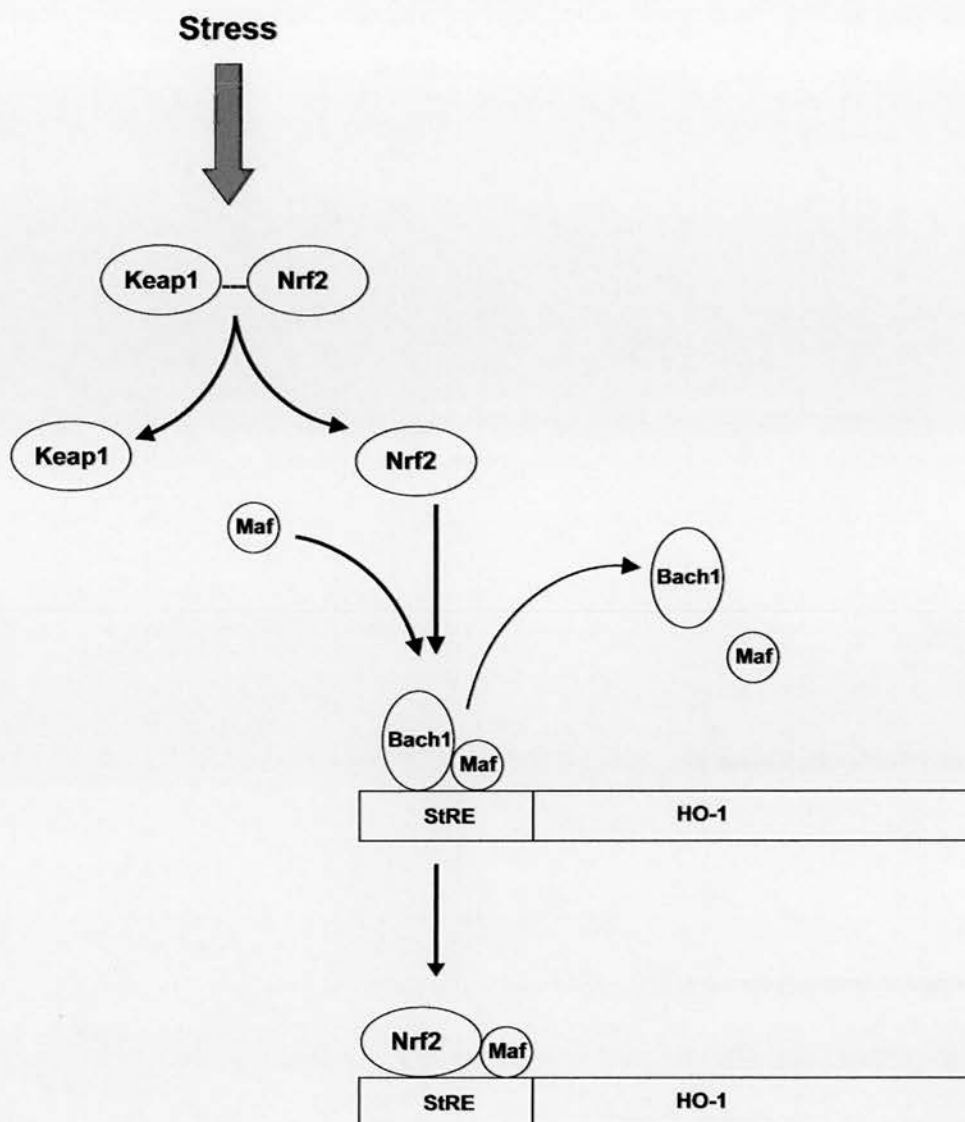


Figure 1.4. Currently accepted model pathway of Nrf2 activation.

Nrf2 is held in an inactive form in the cytoplasm of resting cells by the inhibitor Keap-1. Following a stressful stimulus, these factors dissociate, and Nrf2 migrates to the nucleus. Nrf2 then associates with small Maf proteins and binds to the stress response element in the promoter region of the target gene (**StRE**). In resting cells, this element is inhibited by the binding of Bach1. Bach1 repression is alleviated following stress, allowing Nrf2 to bind.

1.5.2.2 HIF-1 α

Hypoxia is a potent inducer of HO-1 and this appears to be through the activation of Hypoxia Inducible Factor 1 α (HIF-1 α) (Lee 1997, Kacini 2000). The relevant upstream binding sequence in the promoter has been termed the HIF-1 α Response Element (HRE). Whilst the human HO-1 promoter contains an HRE, it has been established that hypoxia does not induce HO-1 in human tissues, although it does in other species (Kitamuro 2003).

Of note, however, cobalt chloride (CoCl₂) which has been used as a hypoxia mimicking agent and a HIF-1 α stimulating chemical, does still induce HO-1 in human cells (Gong 2001). This potentially misleading finding appears to be the result of changes in Bach1, a negative regulator of HO-1 activation (see section on Nrf2). Bach1 is induced by hypoxia in human cells, preventing the induction of HO-1 by hypoxia. Treatment with CoCl₂, however, decreases Bach1 levels, thus inducing HO-1 (Kitamuro 2003).

1.5.3 Functional Importance of HO-1

HO-1 has been found to be a cellular protectant in virtually all tissues of the body, ranging from neurons to muscle cells, and cells of all types of origin. The observation that HO-1 deficiency leads to a chronic inflammatory state in both mice and humans (Poss 1997, Yachie 1999) reinforces the essential nature of HO-1 activity. HO-1 is critically important in human liver, both in maintenance of

homeostasis under physiological conditions, and also during liver injury. The following sections examine the role of HO-1 in normal and diseased liver, and in hepatic transplantation. Finally the role of HO-1 in other transplantable solid organs will be described.

1.5.3.1 Heme Oxygenase in the Liver

Heme oxygenase 1 has a normal homeostatic role in the liver, as shown by the relative abundance of hepatic HO activity, being twice that seen in the kidney, and only inferior to that seen in the spleen and bone marrow (Tenhunen 1970). The parenchymal and sinusoidal cells appear to have disparate roles, with sinusoidal cells destroying senescent red blood cells, and hepatocytes responsible for the degradation of free and haptoglobin-bound haemoglobin (Bissell 1972). Consistent with this, there is differential expression of HO isoforms in the resting liver, with 85% of HO activity being contributed by HO-2 in the hepatocytes. In contrast, hepatocytes constitutively express minimal levels of HO-1, although Kupffer, endothelial and stellate cells all express HO-1 at high levels, but with lower levels of HO-2 than that found in the hepatocytes (Bauer 1998, Rensing 1999).

Bauer's study also examined the pattern of HO-1 induction in the liver in response to various stressors. Whilst all conditions studied led to increased HO-1 expression, different stimuli produced different patterns of induction: LPS induced HO-1 in the sinusoidal lining cells; GSH depletion increased it in pericentral parenchymal cells; cobalt chloride increased it in periportal hepatocytes; haemorrhage and resuscitation

induced HO-1 in both pericentral hepatocytes and sinusoidal lining cells. This may reflect the spatial concentration of reactive oxygen species within the liver in these different models, and is consistent with a common pathway of intracellular ROS formation that induces HO-1 (Bauer 1998). This is also in agreement with studies showing that primary rat hepatocytes subjected to anoxia and re-oxygenation appear to require ROS to induce HO-1 (Ohlmann 2003).

Heme oxygenase 1 has been demonstrated to be protective in the liver in different types of injury. Induction of HO-1 prior to the onset of hepatic inflammation will protect hepatocytes from CD95, LPS and TNF α induced apoptosis (Sass 2003). This protection can be replicated by HO-1 gene transfer, and appears to be primarily due to an effect in the hepatocytes, and is independent of Kupffer cells, with the protection still being apparent in the presence of Kupffer cell depletion (Sass 2003).

1.5.3.2 HO-1 in Liver Transplantation

In the context of liver transplantation, the organ sustains significant ischaemia-reperfusion injury, which is exacerbated by cold storage and the immunological assault following re-implantation. Heme oxygenase has a physiological role in liver transplantation, and has been found to be significantly elevated after graft implantation, particularly being induced in non-parenchymal cells (Kobayashi 2005).

HO-1 induction could improve outcomes after transplantation. Adenoviral HO-1 gene transfer improves graft survival in a rat liver transplant model (Ke 2002). The

various different functions of the three end products of HO-1 activity are likely to each contribute to this. For example, the apoptosis seen in IRI is mediated mostly through ROS generation, which in endothelial cells is iron-mediated (Rauen 1999). HO-1 induction results in increased transferrin as well as synthesis of an iron-exporting pump which will lower intracellular iron levels (Eisenstein 1991, Ferris 1999). Biliverdin and bilirubin are potent antioxidants (Clark 2000, Soares 2004), and bilirubin inhibits ROS induced hepatocyte death (Granato 2003). Furthermore, in models of *ex vivo* perfusion and liver transplantation, biliverdin treatment resulted in improved hepatic function and survival after transplant (Fondevila 2004). CO activates p38 MAPK, which can be anti-apoptotic (Brouard 2000), and in liver transplant models, the beneficial effect of HO-1 gene transfer can be mimicked by chemical delivery of CO by methylene chloride (Ke 2002). In keeping with this, in a rat model of *ex vivo* perfusion, exogenous CO was capable of significantly improving the function of liver grafts, (Amersi 2002).

The potential clinical use of HO-1 manipulation in liver transplantation was demonstrated by the successful transplantation of steatotic livers from fatty Zucker rats. These rats are genetically obese with fatty infiltration of the liver, a frequent reason for non-retrieval or non transplantation of livers from human donors due to the high rate of primary non-function. Pharmacological HO-1 upregulation or HO-1 gene transfer prior to transplantation significantly improved all measured endpoints and improved graft function (and animal survival) from 40% to 80% (Amersi 1999).

Other difficulties with liver transplantation include the limited permissible preservation time. Preservation of liver allografts for longer than 12 hours risks higher rates of primary non-function and poorer long term outcome (Adam 1992). In rats, induction of HO-1 will allow the successful transplantation of liver grafts following 44 hours of preservation, a duration which would otherwise be universally fatal (Redaelli 2002).

Livers are often unusable because the donor is older, which results in poorer long-term graft function. Tullius demonstrated that HO-1 induction with cobalt protoporphyrin was capable of improving long term function of grafts taken from older, “marginal” donor rats from 40% to 80% survival at 24 weeks (Tullius 2002). Thus, if reproducible in humans, HO-1 induction may enable the use of organs that are currently being discarded, and so increase the number of available organs for liver transplantation.

Whilst HO-1 can modulate the peri-transplant events, minimising cell death and reducing primary non-function, chronic rejection is the current main cause of late graft loss (Libby 2001). This is characterised by arteriosclerosis, intimal hyperplasia and interstitial fibrosis, and is associated with activation of endothelial cells, graft infiltration with leucocytes and abnormal proliferation of the vascular smooth muscle cells. Early HO-1 induction may be able to prevent or abrogate these long-term changes.

Endothelial cell loss at the time of transplantation predisposes the graft to late rejection. In a model of chronic rejection in aortic allografts, gene transfer with HO-1 at the time of transplantation reduced intimal hyperplasia and leucocyte infiltration (Chauveau 2002), and this effect can be mimicked by delivery of exogenous carbon monoxide (Chauveau 2002, Otterbein 2003). HO-1 can prevent apoptosis of endothelial cells (Brouard 2000), thereby maintaining the endothelial barrier, and limiting activation of adjacent endothelium. It is likely that HO-1's suppression of chronic rejection is primarily effected by preventing endothelial injury at the time of reperfusion, although it may also have long term effects.

1.5.3.3 Heme Oxygenase in Other Transplantable Organs

Because transplantation provides a useful clinical model of ischaemia reperfusion injury, the effect of heme oxygenase has been examined in many models of transplantation. The kidney is the most commonly transplanted solid organ, and hence would be the candidate for greatest benefit if HO-1 induction were to be shown to be beneficial. The kidney constitutively expresses HO-2, mainly within the tubules and arterioles, whilst basal expression of HO-1 is relatively low (Da Silva 2001). However, when subjected to an appropriate stimulus, the kidney is capable of increasing its HO activity up to four fold, by the induction of HO-1 (Da Silva 2001).

It has been observed that the level of HO-1 expression in the endothelial and smooth muscle cells of cardiac transplants correlates with the continued survival of the graft (Soares 1998), thus providing a physiological role for HO-1 in heart transplantation.

Furthermore, induction of HO-1 prior to graft harvesting, and the attendant ischaemia reperfusion injury, protects the heart from transplant associated injury, as measured by the continued function of the grafts (Akamatsu 2004). This effect was fully reproducible by extrinsic CO administration either to the donor or to the graft during cold storage.

Pancreatic islet cell transplantation is a relatively new form of transplantation, where rather than transplanting the whole pancreas to treat diabetes, only pancreatic islets are transplanted, which contain the β cells which secrete insulin. Recent research has shown that this is a viable method and that the medium term results are acceptable (Shapiro 2000) and use of this technique is likely to increase in the coming decade. However, large numbers of donor islets are required, often necessitating the use of several organs for one recipient to gain an acceptable result. This may be due to islet cell apoptosis during the harvesting and transplantation. Experiments in rat islets have shown that HO-1 can be induced with several agents, including IL-1 β , nitric oxide, cobalt protoporphyrin and cobalt chloride (Ye 1998), suggesting the possibility of HO-1 induction as a strategy to reduce the requirement for large numbers of donor islets, allowing more patients to be treated with fewer organs. Carbon monoxide has been shown to improve the function and survival of grafted β cells in a murine model, demonstrating that enhanced HO-1 activity could have a therapeutic role (Gunther 2002).

1.6 Potential Pharmacological Preconditioning Agents

There are many ways in which to achieve a preconditioned phenotype, as described earlier in this chapter. Although ischaemic preconditioning has been used with some success, this exerts a global effect on the organ, and may result in undesired effects on the liver. Furthermore it can only be applied at the time of surgery. It is for this reason that there is interest in developing pharmacological treatments which can precondition the liver. A pharmacological agent has the advantage that it may work through a specific cellular pathway. There are many potential agents that could be used and have been studied in a variety of *in vitro* and *in vivo* models (Selzner 2003). This work studied in detail the effects of curcumin and cyclosporin A on human hepatocytes.

1.6.1 Curcumin

Curcumin is a natural extract of turmeric, the spice produced from *Curcuma longa* L (Zingiberaceae), part of the ginger family. This herb originates from the tropical regions of Asia, and has been used for many centuries as a herbal remedy for several complaints. In ancient Hindu texts it is used to treat sprains and swellings, and in Indian medicine it is traditionally used for biliary disorders, sinusitis and diabetic wounds (Ammon 1991).

Over the past few decades there has been a great volume of research performed on curcumin to establish its effects at the cellular level and to investigate its potential as

an *in vivo* therapeutic agent for a wide variety of conditions. In vitro and in vivo studies have shown that curcumin is able to induce the stress response (Dunsmore 2001) and to reduce the severity hepatic injury in several models of acute inflammation (Park 2000, Gaddipati 2003), demonstrating that it may have potential as a hepatic preconditioning agent.

1.6.1.1 Cellular Effects

At the cellular level, work has focussed on the effect of curcumin on transcription factors and cell signalling cascades. Curcumin has been shown to inhibit NF κ B in a variety of cell types including macrophages, colorectal and prostatic cell lines and endothelial cells (Pan 2000, Plummer 1999, Mukhopadhyay 2001, Kumar 1998). This effect appears to be the result of IKK inhibition, mediated by inhibiting signalling upstream of IKK (Jobin 1999, Pan 2000, Plummer 1999), and is the likely mechanism through which curcumin reduces a variety of proteins such as ICAM-1, ELAM-1, VCAM-1, and COX-2 (Kumar 1998, Plummer 1999). Curcumin also inhibits AP-1, which in certain cell lines may be a result of JNK inhibition (Jobin 1999, Singh 1995).

Curcumin has been found alter the activity of several cell kinases in particular the MAP kinases. These effects are complex and appear to vary between cell types, although this may be due either to different experimental conditions or to intrinsic differences in the cell lines (table 1.1).

1.6.1.2 Redox Effects of Curcumin

Curcumin has been described as having both pro-oxidant and anti-oxidant effects, and this has been linked to its effects on either producing protection from oxidative stress, or in causing apoptosis. In a model of rat forebrain ischaemia-reperfusion, curcumin administered after the onset of ischaemia reduced xanthine oxidase activity, superoxide anion production and malonaldehyde levels, demonstrating anti-oxidant effects in this model (Ghoneim 2002). Interestingly, although curcumin increases glutathione levels in Jurkat cells (Piwocka 2001), it did not prevent the reduction of glutathione seen in the rat forebrain IRI model (Ghoneim 2002). In contrast to these anti-oxidant properties, the induction of apoptosis by curcumin demonstrated by several groups is associated with the generation of reactive oxygen species (Khar 2001, Woo 2003, Ligeret 2004). These apparently conflicting results may be explained by the different concentrations of curcumin used in these studies, and in the relative sensitivity of different cell lines. Furthermore, other “anti-oxidants” such as the garlic extract DAS have been shown to induce ROS generation (Gong 2004). Providing that the cell is not overwhelmed by ROS, it is the response to this stimulus by the production of protective proteins that may mediate the “anti-oxidant” properties of these compounds (Gong 2004).

Curcumin predominantly induces apoptosis through a classical mitochondrial pathway, associated with mitochondrial pore opening, swelling, cytochrome C release and caspase activation (Anto 2002, Woo 2003). Atypical variants have been described where chromatin clumping occurs, but there is no change in caspase



activity, no mitochondrial depolarisation and glutathione depletion does not occur (Piwocka 1999 and 2001). Curcumin can also promote apoptosis by inhibiting Bcl-xl through NFκB inhibition (Han 1999). Indeed, NFκB inhibition may be critical as its over-expression can prevent curcumin induced apoptosis (Anto 2000).

Curcumin-induced apoptosis only occurs in certain cell lines. These include hepatocellular, colonic, reticuloendothelial and renal cells (Khar 2001, Woo 2003). Cell lines which undergo apoptosis generate high levels of superoxide when treated with curcumin, and the apoptosis can be prevented by the addition of the free radical scavenger N-acetyl-cysteine (NAC) (Morin, 2001, Khar 2001, Woo 2003). Superoxide may not explain all of the effects of curcumin, as in isolated rat mitochondria, curcumin is capable of lowering superoxide levels, whilst also inducing HO[•] production by facilitating the Fenton reaction due to its iron reducing capabilities (Ligeret 2004).

Cell Type	P38	JNK	ERK	Reference
Intestinal epithelial	↑			Jobin 1999
Renal epithelial	↑	↔	↑	Balogun 2003
Breast cancer	↔	↓	↓	Squires 2003

Table 1.1 Varying effects of curcumin on MAPKs in different cell types.

1.6.1.3 Curcumin and the Stress Response

Curcumin has been shown to induce Hsp70 in HeLa cells and colorectal cell lines (Dunsmore 2001, Chen 2001). This has been associated with activation of HSF-1. However, these reports are inconsistent. Chen showed nuclear localisation of HSF-1, and increased binding to the HSF-1 consensus sequence, but on cross linking studies the HSF-1 was not trimeric following curcumin treatment, suggesting that the HSF-1 would not be transcriptionally active, and may not be responsible for the observed rise in Hsp70 (Chen 2001). However, Dunsmore found that Hsp70 was clearly induced by curcumin, and in HSF-1 $-/-$ fibroblasts, the induction was markedly reduced, implicating the involvement of HSF-1 (Dunsmore 2001). In rat hepatocytes, Kato found that curcumin had no effect on Hsp70 levels when given alone, although it did potentiate the effects of other Hsp inducers, such as heat shock and arsenite (Kato 1998).

Curcumin is also capable of inducing HO-1 in a variety of cells (Scapagnini 2002, Motterlini 2000, Balogun 2003). This may be mediated through Nrf2 activation, which binds to the ARE sequences in the HO-1 promoter (Balogun 2003). Of note, curcumin does not induce HO-1 in HeLa cells, whilst it does induce Hsp70 (Dunsmore 2001). Thus the effects of curcumin may be cell type dependent.

1.6.1.4 Clinical Application of Curcumin

The impetus for much of the research into curcumin has been the potential to apply it in a therapeutic role. It was historically used to treat inflammation and swelling, and early research focussed on these effects, establishing that curcumin reduces inflammation in the rat paw oedema model, and in formalin induced arthritis (Ammon 1991). This was extended into other models of organ damage, and curcumin has proven effective in reducing liver injury from carbon tetrachloride (Park 2000), aflatoxin B (Soni 1992), cyclophosphamide (Soudamini 1992) and haemorrhage/resuscitation injury (Gaddipati 2003). It reduces the severity of renal ischaemia-reperfusion injury (Shoskes 1998), and is protective in *in vitro* models of endothelial cell injury (Motterlini 2000).

The protective effects of curcumin may be due to the modulation of MAP kinase cascades (Balogun 2003), or to the induction of stress proteins such as Hsp70 and HO-1 (Dunsmore 2001, Scapagnini 2002). It should be noted, however, that there are many other proteins which are induced by curcumin treatment which may also contribute to the protection seen in the above models such as induction of the phase II detoxifying enzymes glutathione S transferase and NADPH:QR (Okada 2001). Hence the results available demonstrate that curcumin has potential preconditioning properties, although this has not previously been tested in human hepatocytes.

1.6.2 Calcineurin Inhibitors

Calcineurin inhibitors have been the mainstay of immunosuppressive therapies since Roy Calne first demonstrated the clinical use of cyclosporin A (Calne 1981). This was added to by the later development of tacrolimus, another calcineurin inhibitor. Whilst these are structurally different, and originate from different sources, they both function to inhibit the protein phosphatase calcineurin. Classically, their immunosuppressive effect has been linked to inhibition of calcineurin and subsequent downstream prevention of IL-2 production and T cell proliferation. However, in recent years, these drugs have been found to have many other cellular effects and may have the potential to be used in other clinical arenas.

1.6.2.1 Calcineurin

Calcineurin is a serine/threonine protein phosphatase which is essential for production of IL-2, an important factor for T cell proliferation. It was initially purified from mammalian brain (Klee 1988), and was identified as being dependent on calcium and calmodulin as co-factors in its activation, and hence the name calcineurin, although it has also been known as protein phosphatase 2B.

Calcineurin is a heterodimer of two different subunits – A and B. Calcineurin A contains the catalytic region, a calmodulin binding domain and an autoinhibitory domain, whilst calcineurin B contains the calcium binding domain. The

autoinhibition is only overcome in the presence of both calcium and calmodulin, allowing the phosphatase function to commence (Rusnak 2000).

Amongst other effects, calcineurin activity dephosphorylates the cytoplasmic subunits of the transcription factor Nuclear Factor of Activated T cells (NF-AT), resulting in its nuclear migration and DNA binding, leading to increased IL-2 synthesis (Rao 1997).

1.6.2.2 Cyclosporin A

Cyclosporin A was purified from the fungus *Tolypocladium inflatum*. It is a lipophilic molecule which can easily enter the cell, where it binds to cyclophilin. This is an immunophilin which acts as a cytoplasmic receptor for cyclosporin. It is found in a wide variety of cells and species, and is characterised by chaperone-like activity aiding the folding of ribonuclease, although this does not appear to be related to the immunosuppressive effect of cyclosporin (Ruhlmann 1997).

1.6.2.3 Tacrolimus

Tacrolimus was isolated from *Streptomyces tsukubaensis*. Structurally it is a macrolide, and it is also known as FK506. It was first discovered in 1984, and the first experimental results were published in 1987 (Wallemacq 1993). Like cyclosporin A, it is also lipophilic and enters the cell to bind to immunophilins which are termed FK506 Binding Proteins (FKBPs). These have the same enzymatic

activity seen in cyclophilin, allowing tacrolimus to interact with and inhibit calcineurin (Dumont 2000).

1.6.2.4 Mechanism of action of Calcineurin Inhibitors

Despite the structural differences of tacrolimus and cyclosporin, their different origins and their different cytosolic receptors, they both inhibit the action of calcineurin. The drug-immunophilin complex interacts with the catalytic domain of calcineurin, blocking its phosphatase activity. This results in the inability of the cell to activate NF-AT and hence prevents upregulation of IL-2 production, which is believed to be central to the immunosuppressive effects of these drugs (Beals 1997).

Calcineurin has a wide range of other physiological functions ranging from osteoclast bone resorption to regulation of pituitary hormone secretion (Rusnak 2000). Thus calcineurin inhibitors will have effects other than those on NF-AT activity. Calcineurin inhibitors stimulate the expression of several cytokines and growth factors, including TGF- β , IFN- γ , and IL-13. (Shin 1998, Rafiq 1998, van der Pouw Kraan 1996). TGF- β has potent immunosuppressive effects, and much of the effects attributed to cyclosporin may be through its stimulation of this cytokine (Khanna 1999).

1.6.2.5 Calcineurin Inhibitors and the Stress Response

Calcineurin inhibitors also affect stress protein production. The transcriptional activity of HSF-1 is tightly regulated by multiple repressive factors including association with Hsp90 and Hsp 70 (see section 1.4.6). The association with Hsp90 is dependent on the presence of Cyp40, a cyclophilin (Duina 1998). Calcineurin inhibitors will displace Cyp40, potentially resulting in activation of HSF-1.

Furthermore Hsp70 can bind to cyclosporin (Moss 1992). Thus cyclosporin could derepress HSF-1, facilitating its induction in response to a stimulus. Consistent with this mechanism, cyclosporin treatment of HeLa cells produces a degree of HSF-1 and HSF-2 activation in resting cells, and also leads to a greater activation of HSF-1 in response to heat shock (Paslaru 2000). In rat hepatocytes, cyclosporin also leads to a dose-dependent activation of HSF-1 (Andres 2002). The mechanism of HSF-1 activation by cyclosporin has not been precisely delineated, but it may be due to ROS generation (Andres 2002), or to inhibition of the proteasome (Paslaru 2000)

In keeping with its effect on HSF-1, cyclosporin is capable of inducing Grp78 (an ER chaperone) and Hsp27 (Paslaru 1994, Paslaru 2000). The effect of tacrolimus on HSF-1 has not been directly tested, but it has different effects on Hsp production in comparison to cyclosporin. Both tacrolimus and cyclosporin induce Hsp70 in rat hepatocytes (Kaibori 2001, Andres 2002). In contrast, tacrolimus has no effect on Grp78 expression in HeLa cells, whilst cyclosporin strongly induces it.

Calcineurin inhibitors also have effects on other transcription factors and kinase pathways which are relevant to any potential preconditioning effects. Cyclosporin has been found to affect the activity of AP-1 and NF κ B (Mattila 1990, Rincon 1994, Andres 2002). CsA can also activate ERK 1 and 2 (Paslaru 1997). Cyclosporin inhibits JNK and p38 pathways in T cells (Matsuda 1998), as does tacrolimus (Matsuda 2003).

Regulation of mitochondrial events is important in the response of cells to stress. The collapse of the mitochondrial membrane potential is a key component of cell death in ischaemia-reperfusion injury, and results from the development of the mitochondrial permeability transition pore (see section 1.2.1). This pore contains several protein subunits which are assembled at the contact point between the inner and outer mitochondrial membrane to create an opening (Marzo 1998). Mitochondrial cyclophilin D is a key constituent of the pore and cyclosporin treatment prevents its recruitment to the pore, inhibiting pore formation, preventing cell death (Pastorino 1993, Halestrap 1997). The combined effect of upregulation of Hsps and inhibition of the mitochondrial permeability transition pore suggests that cyclosporin may have cytoprotective effects which make it a candidate preconditioning agent.

1.6.2.6 Preconditioning Effects of Calcineurin Inhibitors

The understanding of the effects of calcineurin inhibitors on the stress response has led to interest in using them as preconditioning agents. Cyclosporin ameliorates the degree of liver injury in a rat IRI model (Konukoglu 1998). This effect was achieved by administering the drug at the time of reperfusion and as such is unlikely to be the result of *de novo* protein synthesis. There was a lower level of lipid peroxidation in the treated group, suggesting that this preconditioning effect may be due to inhibition of ROS production.

In a rat model of renal ischaemia reperfusion injury, pretreatment with either cyclosporin or tacrolimus was able to reduce the degree of reperfusion injury (Yang 2001). In this model, the dose and timing of the drugs was designed for the ischaemic injury to be applied at the time of maximal Hsp70 expression. Thus the preconditioning effect in this experiment may have been due to increased Hsp levels.

An *in vitro* model of IRI in cardiomyocytes found that preconditioning with tacrolimus improved cell survival, but that cyclosporin did not have the same effect (Cumming 1996). This study allowed time for new protein synthesis (18 hours between pretreatment and the injury) but no difference was found in the expression of the various putative proteins examined (Hsp72, Hsp60, Hsp56, Hsp90).

Calcineurin inhibitors can also protect from other types of injury. Pretreatment with tacrolimus protects rat livers from cold ischaemia (Okano 1994). Pretreatment of the

recipient with cyclosporin improved survival after liver transplantation, although donor pretreatment did not have the same effect. Of note, pretreatment was given for 3 days in both groups (Kai 1994).

Hence there is sufficient evidence to implicate cyclosporin as a candidate preconditioning agent which may be effective in human hepatocytes, and thus is worthy of further study.

1.7 Conclusion

Liver surgery and transplantation is an area of clinical practice in which preconditioning treatments could have a beneficial effect. Such treatments could reduce perioperative hepatic impairment following elective liver resection, or primary non-function following transplantation. The damaging effect of both of these clinical situations reflects the ischaemia-reperfusion injury that is an unavoidable part of the surgical process. This is characterised by a complex series of harmful cellular events. The stress response is part of the natural defence mechanisms of the cell, and it acts to limit the severity of this injury. Preconditioning can upregulate this response prior to surgery, resulting in protection of the liver cells from injury. There are many aspects of the stress response which could be targeted to achieve preconditioning, and current evidence suggests that curcumin and cyclosporin may both be candidate preconditioning agents. However, there is limited knowledge of their effects in human hepatocytes.

1.8 Hypotheses

1. Curcumin induces the stress response in human hepatocytes.
2. Curcumin exerts a preconditioning effect on hepatocytes that protects them from ischaemia-reperfusion injury.
3. Cyclosporin A induces the stress response in human hepatocytes.
4. Cyclosporin A preconditions human hepatocytes and protects them from ischaemia-reperfusion injury.
5. Induction of the preconditioned phenotype does not impair differentiated cell function in human hepatocytes.

Chapter 2

Materials and Methods

2.1 Cell Culture

All cell cultures were performed in standard conditions of 37°C, 5% CO₂. HepG2 (Morris 1982) and Huh7 (Nakabayashi 1982) human hepatoma cell lines were obtained from the European Tissue Culture Collection (Porton Down, UK). Both lines were maintained in Dulbecco's Modified Eagle's Medium containing 10% fetal calf serum, 50u/ml penicillin G and 50µg/ml streptomycin. Cells were trypsinised and passaged twice weekly in 75cm² flasks. Plating densities in different dishes are shown in table 2.1. Cells were used for experiments at 60 to 80% confluence.

2.2 Primary Hepatocyte Isolation

Normal liver tissue was collected at the time of hepatic resection from patients who had given preoperative written consent. Local ethical approval had been obtained (LREC reference number LREC/2000/5/23). Preparation of hepatocytes was essentially as described previously (O'Riordain, 1999). Approximately 20g of liver tissue was cut from the operative specimen following removal from the patient. All visible vessels were irrigated immediately with sterile ice cold heparinised saline (10u/ml) until the flow through was clear. The sample was then transferred to the laboratory in ice cold saline. All following steps were performed within a laminar flow cabinet. Cannulae (16G venflons) were inserted into at least 4 different vessels, sutured in place and connected to tubing via a pump. Unused open vessels were closed with sutures. The specimen was placed on a sterile container dish floating in a

	96 well plate	12 well plate	35mm dish/ 6 well plate	10cm dish	75cm ² flask
Cell Number	2×10^4	1.5×10^5	3×10^5	2×10^6	3×10^6
Medium Volume	100μl	1ml	2ml	10ml	17ml

Table 2.1 Culture conditions of cells in different dishes

water bath set at 37°C, and perfused with sequential oxygenated buffers A to C warmed to 37°C in a separate water bath. Oxygenation was achieved by bubbling medical oxygen through the buffer at 1l/min. Waste buffer was continuously drained from the container by a siphon drain. Buffer D, containing collagenase and hyaluronidase was perfused through the tissue for 1 hour at 37°C. During this time the drain was removed from the container and the buffer was continuously recirculated. Following digestion the liver capsule was teased apart and hepatocytes collected by irrigating with serum free medium (William's E, with penicillin and streptomycin). The suspension was passed through a 200 micron mesh filter, and then centrifuged 3 times at 50g for 2 minutes at 4°C. The pellet was retained and resuspended in medium following each centrifugation. Following washing and centrifugation, the cells were gently mixed with equal volumes of a 100% Percoll gradient and centrifuged at 50g for 10 minutes at 4°C. The pellet was then resuspended in medium containing 10% fetal calf serum and the cell number counted using a graticule. The percoll gradient removes dead hepatocytes, and results in a highly purified population. Cells prepared in this way contain no CD14 positive staining cells on flow cytometry and have a final viability as tested with trypan blue of >98% (O'Riordain 1999). The yield of each preparation varied from 3 million to over 20 million hepatocytes depending on the donor. The cells were plated out in collagen coated (Worthington, 5µg/cm²) dishes, and left overnight to adhere. Experiments were performed the following day. The cells were maintained in William's Medium containing 10% fetal calf serum with 50u/ml penicillin G and 50µg/ml streptomycin as above.

2.3 Liver Tissue Slices

Normal liver tissue was collected at the time of hepatic resection from patients who had given preoperative written consent. A small cube of liver tissue was collected in theatre under aseptic conditions and transferred to the laboratory in ice cold saline. This was placed in a sterilised custom built clamp and slices were cut by hand to coverslip thickness using a skin graft blade. These slices were then placed in 35mm tissue culture dishes containing 2mls Williams' medium with 10% FCS and incubated in standard conditions (37°C, 5% CO₂).

2.4 Immunohistochemistry

Sections were cut from paraffin embedded tissue and mounted on APES (α -propyl ethoxy silane) coated glass slides. The sections were initially dewaxed in xylene for ten minutes and then rehydrated by 2 minutes washes in stepped alcohols, decreasing from 100% to 50% alcohol. Endogenous peroxidase activity was blocked by incubating the slides in 3% hydrogen peroxide in water for 15 minutes. The slides were then rinsed in distilled water followed by 5 minutes incubation in TBS. Subsequently antigen retrieval was performed. The slides were placed in citrate buffer in a sealed plastic container and then microwaved for three 5 minute periods (800W microwave on full power). The slides were rotated in the container after each period, and then left to stand for twenty minutes in hot buffer.

The slides were washed twice in TBS for 5 minutes and then appropriate blocking serum was applied, diluted 1 in 5 in TBS and incubated for 20 minutes. The serum selected was from the same species in which the secondary antibody was raised. The blocking serum was then tapped off and the slide dried prior to application of the primary antibody, which was then incubated for either 1 hour at room temperature or overnight at 4°C. The slides were washed three times in TBS for 5 minutes, after which the secondary antibody was applied and incubated at room temperature for 30 minutes. The slides were washed as before and then incubated with diaminobenzidine solution (DAB), prepared as per the manufacturers instructions (Sigma Fast, Sigma). The slides were then rinsed in water and counter-stained with haematoxylin for twenty seconds, rinsed in running water, blued in Scott's Tap Water Substitute for one minute and rinsed in water. Finally the slides were dehydrated by consecutive rinsing in ascending alcohols and cleared in xylene. They were then mounted with a coverslip using DPX mountant and left to set overnight.

If a biotin conjugated secondary antibody was used, endogenous biotin was blocked using a commercially available kit (Vector Laboratories), which was applied to the slides prior to the primary antibody. Avidin D was applied and incubated for fifteen minutes, rinsed off and then biotin blocking solution was added to the slides and incubated for a further fifteen minutes. The slides were then treated with primary and secondary antibodies as above. However, prior to application of the DAB solution, there was a thirty minute incubation in streptavidin-biotin-HRP complex (DAKO) following which the slides were then rinsed again and the DAB applied. All other steps were as described above.

2.5 Viability Assays

Cell viability assays were performed on using a modification of an established MTT (methylthiazole tetrazolium) dye technique (Takahashi 2002). This method relies on the reduction of tetrazolium salts by mitochondria, generating formazan crystals. It is a measure of cell viability and may be more sensitive the lactate dehydrogenase in detecting impaired cell function (Liu 1997, DaCosta 1999). The formazan crystals are then solubilised with SDS, and the concentration is read spectrophotometrically. Cells were cultured in 96 well plates at 2.5×10^4 cells per well in 100 μ l of medium. Following the requisite treatment, 10 μ l of MTT solution (5mg/ml in DPBS) was added to each well and the plate returned to the incubator for 4 hours. Subsequently, 100 μ l of extraction buffer (10% SDS, pH 3) was added and the plate incubated at 37°C overnight, following which the optical densities were measured at 570nm using a plate reader (Dynex MRX II, Dynatech).

2.6 Cell Death Assays

Assessment of cell death was performed by measuring lactate dehydrogenase release in cell culture supernatants, as described by Bergmeyer and Bernt (Bergmeyer 1974). This method results in the generation of formazan crystals, the concentration of which is then measured spectrophotometrically. Following the treatment of cells, an aliquot of supernatant was collected and frozen at -70°C for later analysis. To perform the assay, 10 μ l of supernatant was pipetted into a well in a 96 well plate,

and 20µl of tris buffer (1M Tris-HCl, pH 8.5) and 50µl of substrate (0.1mM l-lactate, pH5.5) were added. The plate was warmed for five minutes in an incubator at 37°C and then 20µl of chromogen (5mg/ml NAD, 2mg/ml INT, 0.5mg/ml PMS) was added and the plate returned to the incubator for exactly five minutes before 100µl of 0.5M HCl was added to each well to stop the reaction. The plate was read within 20 minutes, in a plate reader at 540nm. All reactions were performed in triplicate and the mean value obtained. Blanks were obtained by substituting 10µl of blank (0.01M sodium oxalate) solution in place of a supernatant. All values were expressed as a ratio of control samples, which were obtained from untreated cells cultured at the same time and in the same conditions as the treatment group, and which had the LDH assay performed on the same plate.

2.7 Models of Hepatocellular Injury

Several different models of cell injury were developed, as described in chapter 6. Three are described here in detail: hydrogen peroxide/glucose oxidase, sodium arsenite and reperfusion after cold storage. Other methods that were tested during model development are described in chapter 6.

2.7.1 Hydrogen Peroxide/Glucose Oxidase

Cells were cultured in 96 well plates and pretreated as required. Subsequently the were challenged with medium containing either 1mM hydrogen peroxide for four

hours or with 75-100mU glucose oxidase for two hours. An MTT assay was then performed as described above. Results were expressed as percentage protection, which is calculated by the following formula: $((X/H_2O_2 \text{ group})-1) \times 100$, This represents the improvement in survival over the group which had received no pretreatment. Each experiment contained an untreated group for such a comparison.

2.7.2 Sodium Arsenite

Initial experiments with sodium arsenite used an MTT assay as the end measurement of survival, and well performed in a 96 well plate format, as described above. This was subsequently altered to a 6 well plate format using flow cytometry to assess survival, as follows. Cells were cultured in 6 well plates and pretreated as required. The cells were then treated with 1 to 10mM sodium arsenite for times ranging from thirty minutes to two hours. The cells were recovered in normal medium for 18 hours and then harvested for flow cytometry using propidium iodide and annexin V

To ensure floating cells were included, the medium was aspirated from each well and placed in a LP3 tube. From this point on, the tube was kept on ice unless otherwise stated. The well was washed with 200µl of warm DPBS, which was then aspirated and added to the tube. 200µl of warmed trypsin was then added to each well, and the plate replaced in the incubator at 37°C. The plate was checked every minute, and removed from the incubator when it was seen that the cells came off the plate when it was gently tapped. 200µl of ice-cold DPBS was then added to the well and then the well was aspirated and added to the LP3 tube. A further 200µl wash was performed

and collected. The tubes were then centrifuged at 2,000g for five minutes at 4°C. The supernatant was removed and the cell pellet washed and resuspended in 0.5mls of cold DPBS. Two further washes were then performed.

For dual staining with annexin V and propidium iodide, the Annexin V-FITC Apoptosis Detection Kit (BD Pharmingen) was used as per the manufacturer's instructions. Briefly, the pellet of cells was resuspended in 1x binding buffer at 1×10^6 cells per ml. 100µl of the cell suspension was placed in a fresh tube, and 5µl each of annexin V and propidium were added. The tube was briefly vortexed and then incubated for fifteen minutes at room temperature in the dark. Subsequently, 400µl of 1x binding buffer was added, the tube was placed on ice and read within sixty minutes.

Cells were analysed on an Epics XL-MCL machine (Coulter). Gates were set on initial control cells, which included the following groups: unstained; annexin V only; propidium only; propidium and annexin. These controls were repeated with an injured population. The programme was set to register a either 5,000 or 10,000 events depending on cell numbers.

2.7.3 Cold Storage and Reperfusion

Storage of cells in University of Wisconsin solution (UW) was used to induce cold injury. Cells were pretreated as required and then the medium was removed and replaced with UW at 4°C. The lids of the plates were sealed with parafilm, and then

they were incubated for the required period of time at 4°C. For recovery, the UW was removed and replaced with medium containing 10% FCS, pre-warmed to 37°C, and the plate was replaced in the normal incubator (37°C, 5% CO₂). After the required recovery period, cell injury or survival was assessed by using either measurement of released LDH in the supernatant, or by performing an MTT assay.

2.8 Preparation of cell lysates

2.8.1 Whole cell extracts

Whole cell extracts were obtained using RIPA buffer with protease inhibitors. The medium was removed from the cells and they were placed on ice and washed with ice cold PBS. This was then removed and the well aspirated dry prior to addition of the lysis buffer. The cells were scraped in this buffer and collected in an eppendorf which was then centrifuged at 10,000g for 10 minutes at 4°C. The supernatant was then collected and placed in a fresh tube.

2.8.2 Nuclear and cytoplasmic extracts

Nuclear and cytoplasmic fractionation was performed using Gobert's method (Gobert 1996). The medium was removed from the cells and they were placed on ice and washed with ice cold PBS. This was aspirated to dryness and buffer E was added. The cells were scraped in this buffer and collected in an eppendorf prior to centrifugation at 10,000g for 2 minutes at 4°C. The supernatant containing the

cytoplasmic extract was collected and stored. The pellet was resuspended in buffer F and incubated on ice for 30 minutes prior to centrifugation at 10,000g for 10 minutes at 4°C. The supernatant was then collected and stored as the nuclear extract.

2.8.3 Native whole cell lysates

Cells were washed on ice with PBS and the wells dried as above. Fresh PBS was added to the well and the cells scraped with a cell lifter and collected into an eppendorf. The tube was spun for 10s at 3000g to pellet the cells, and the supernatant was removed. The tube was snap frozen in liquid nitrogen and then thawed on ice. Native lysis buffer was added at twice the pellet volume and the pellet was resuspended and incubated on ice for 30 minutes. The tube was then centrifuged at 10,000g for 15 minutes and the supernatant containing the extract was collected.

2.9 Protein Assay

A protein assay to ascertain the concentration of protein in cell extracts was performed on all samples prior to further analysis. The BioRad DC Protein Assay kit was used according to the manufacturers instructions. Concentrations were established by comparison to a standard curve formed from a known commercially available standard of bovine albumin (BioRad). Plates were read on a Dynatech plate reader (Dynex MRX II, Dynatech).

2.10 Western Blotting

Western blots were performed using acrylamide gels and a standard protocol, with only minor adjustments being made for certain antibodies. Minigels were cast and run using the BioRad Miniprotean II or III systems. Larger gels were cast on the BioRad II xi cell system. Most samples were run on a 10% gel separating gel with a 4% stacking gel. Certain applications required gels ranging from 5 to 20%. For non-denaturing gel electrophoresis to examine the trimeric state of HSF-1, native gradient gels were poured using a specially constructed mixing device based on the commercially available BioRad Gradient Former. When used these gels contained a 3-20% gradient with no stacking gel, and a special non-denaturing sample loading buffer was used.

Protein samples were prepared by the addition of loading buffer and denatured by heating to 95°C for 5 minutes. Samples were then loaded onto the gels and run at 200V until the dye front reached the bottom of the gel. The gel was then removed from its glass plates and placed next to a nylon membrane, in a sandwich of blotting paper. The paper and membrane had previously been soaked in transfer buffer. The sandwich was then placed in a Pharmacia Novablot Semidry transfer blotter and transferred at 0.8mA/cm² for one hour. The membrane was then removed from the sandwich and stained with Ponceau red to examine the protein loading and to ensure adequate transfer. This was then washed out with TBS and then the membrane was washed in blocking solution (5% non-fat milk) for 30 minutes on a rocking platform at room temperature.

Subsequently the membrane was placed in primary antibody at the required concentration and either incubated for one hour at room temperature or a 4°C overnight. The membrane then underwent three five minute washes in TBS-Tween before being incubated in secondary antibody at room temperature on a rocker for 30 minutes. Finally the membrane was washed twice in TBS-Tween (for five minutes each time) and then placed in TBS ready for transfer to the dark-room.

In the dark-room membranes were placed on cards and chemiluminescence was performed using the Amersham ECL system. Membranes were incubated with ECL as per the manufacturer's instructions. The ECL was then removed, the membrane dried with a tissue and covered in Saranwrap and then placed in a cassette with radiographic film (Kodak) and exposed for the required length of time. The films were developed either by hand using traditional developer and fixer chemicals, or using an Amersham Hyperprocessor automated developer.

2.11 RNA Extraction

All reaction compositions are as detailed in Appendix B. RNA was extracted from cultured cells using Trizol reagent (Invitrogen) as per the manufacturer's instructions, and quality and quantity assessed by spectrophotometry. Following the required treatment the medium was aspirated and 1ml of Trizol solution was applied per 10cm² of surface area (1ml for a 35mm dish). The cell lysate was then passed several times through a pipette tip and then incubated at room temperature for five minutes. Chloroform was then added (0.2mls per 1ml of Trizol) and the tubes shaken

vigorously by hand before a three minute incubation at room temperature, followed by centrifugation at 10,000g for 15 minutes at 4°C. The upper aqueous phase was collected in a fresh tube and isopropanol (0.5mls per 1ml of initial Trizol) added before a ten minute incubation at room temperature and then centrifugation as before for ten minutes. The supernatant was removed and the RNA pellet washed once with 75% ethanol, vortexed and then centrifuged at 7,000g for five minutes at 4°C. The alcohol was removed and the pellet air dried for five minutes prior to rehydration in RNase free water. The quantity and quality of the RNA was assessed spectrophotometrically (Ultrospec 2000, Pharmacia Biotech) by measuring the $A_{260/280}$ ratio. Partially dissolved samples with a ratio of less than 1.6 were incubated at 60°C for ten minutes to aid in redissolving the pellet.

2.12 Reverse Transcription

Prior to reverse transcription, the extracted RNA was then treated with RQ1 DNase (Promega) as per the manufacturer's instructions (8µl RNA, 1µl 10x DNase Buffer, 1µl DNase, incubated at 37°C for 30 mins; then add 1µl stop solution and incubate at 65°C for 10 mins). For quality control, an aliquot was then used for a PCR of cytochrome B to confirm the absence of genomic DNA contamination. Reverse transcription was performed using random primers using AMV reverse transcriptase (Promega). A master mix reaction (see appendix B) was prepared and added in equal volume to 10µl of DNase treated RNA, prior to incubation at 42°C for one hour, and

95°C for ten minutes. Confirmation of adequate cDNA was confirmed by PCR for cytochrome B.

2.13 Standard PCR

Specific primers were designed using the primer 3 web-based software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). MgCl₂, Taq polymerase, dNTPs and Taq buffer were obtained from Promega. A reaction mix was prepared containing: MgCl₂, 1xPCR buffer, 1mM dNTPs, 1μM each of the forward and reverse primers, 1μl cDNA, DNase free water and 0.2μl Taq polymerase, to a total reaction volume of 25μl. The concentrations of primers and MgCl₂ were optimised for each set of primers. The thermocycler programme was also optimised for particular reactions.

Following the termination of the thermocycler programme, the PCR products were analysed by electrophoresis on either an acrylamide or agarose gel, depending on requirements. Agarose gels were cast in horizontal gel tanks (Owl Scientific, Woburn, USA). Gel mixtures of 0.8 to 4% agarose were used depending on the size on the product to be run. The agarose was dissolved in 1xTAE buffer and microwaved to dissolve fully. Once set, the gel was bathed in 1xTAE, 10μl of each sample was mixed with 2μl of loading dye (Promega) and added to the wells. The gel was then run at 80V for 30 minutes. A variety of DNA ladders were run alongside

the experimental samples (25bp, PCR markers and 1kb markers, all from Promega) to allow assessment of product size.

If greater resolution was required, acrylamide gels were used. These were cast in the Miniprotean III system (BioRad), and gels of 10 to 20% were used as required. These contained and were run in 1xTBE buffer. Samples and ladders were prepared as above. 10bp ladders (Invitrogen) were used when required. Gels were stained in ethidium bromide after running, and visualised by UV transillumination on a Gel Doc 2000 (BioRad).

2.14 Real Time PCR

Real time PCR was performed using the FRET (fluorescence resonance energy transfer) based Taqman system (Applied Biosystems). Specific primers and probes for each target were designed using Primer Express software version 1.0 (PE Biosystems). Oligonucleotides were then purchased from Applied Biosystems. Probes were labelled with a 5'-FAM reporter and a quencher. Sequences are listed in Appendix B. 18S was used as an internal control and its probe labelled with 5'-VIC and 3'-TAMRA. 2.5µl cDNA was mixed with 100nM primers and probe, and TaqMan Universal PCR Master Mix (Applied Biosystems) to obtain a 25µl reaction. Real Time PCR was performed in 96 well plates with optical caps (Applied Biosystems) and run on an ABI PRISM 7700 real time thermocycler (PE Biosystems). All reactions were performed in duplicate. Serial dilutions of a stock

cDNA from control hepatocytes and negative controls with no template were used to obtain a standard curve, obtained by plotting the dilution factor against the Ct values. Samples generating FAM Ct levels of greater than 36 or VIC Ct levels of greater than 23 were discarded as inaccurate. Relative quantity values were generated as fold difference with respect to the standard curve. Results are expressed as fold difference with respect to matched untreated controls, and corrected for the 18S internal controls. Data analysis was performed using Microsoft Excel.

2.15 Transfections

Transient transfections were performed using Fugene reagent (Roche) as per the manufacturer's instructions, at a ratio of 6 μ l fugene/ μ g plasmid DNA. HUH7 cells were plated at 4x10⁵ cells per 35mm dish, and transfected the day after plating. The standard protocol cultured the cells in 2mls of medium. 6 μ l of Fugene reagent was added to 100 μ l of antibiotic free medium and incubated at room temperature for 15 minutes. 1 μ g of plasmid DNA was then added and incubated for 15 minutes. The mix was then added to the cell culture dishes. All reporter transfections were dual transfections with an appropriate reporter as a control. As a result, the reaction contained 0.5 μ g of each plasmid. The β -galactosidase/Hsp70 reporter construct was a kind gift from WJ Welch (UCSF, USA). The pHOGL3/11.6 reporter construct containing 11.6kb of the human HO-1 promoter was a kind gift from Dr Agarwal, (University of Alabama, USA). The HIF-1 α was a kind gift from Professor Esumi (National Cancer Center, Kashiwa, Chiba, Japan) through Dr Dulak (Jagiellonian

University, Krakow, Poland) PSV- β Galactosidase or pGL3-Control vectors (Promega) were co-transfected with the reporter constructs, to control for transfection efficiency. The cells were treated the following day and the β -galactosidase or luciferase activity assayed using a proprietary kit as per the manufacturer's instructions, following cell lysis in Reporter Lysis Buffer (Promega). Results were calculated as units of luciferase activity per units of β -Galactosidase activity for pHOGL3/11.6 and PSV transfection and vice versa for the Hsp70/ β Gal and pGL3-Control combination.

2.16 Heme Oxygenase Activity Assay

Heme oxygenase activity was determined using a modification of previously published methods (Tenhunen 1968 and 1972, Motterlini 1996, Cuturi 1999), and development of the assay within this laboratory is discussed in appendix A. Following the required treatment, the medium was aspirated from the cells and then the cells were washed once with DPBS. This was then removed, and a small volume of buffer G (100mM potassium phosphate, 2mM MgCl_2 , pH 7.4) added. Cells were then harvested by scraping gently with a cell scraper. The suspended cells were collected, and the dish washed with a further small volume of buffer G, which was also collected. The cells were pelleted by centrifugation at 2,000g for two minutes at 4°C and then resuspended in 220 μ l Buffer G and subjected to three cycles of freeze thawing. A protein assay was then performed as described above to calculate the concentration of protein in the sample. As a source of biliverdin reductase, human

liver cytosol was obtained by homogenising samples of liver in buffer H (0.1M sodium citrate, 10% glycerol, pH 5), and collecting the supernatant following centrifugation at 105,000g for 30 minutes. A 400µl reaction mixture was then created, containing: 500µg of cell protein; 1.5mg liver cytosol; 0.8mM NADPH; 2mM glucose; 6 phosphate; 0.2 units glucose 6 phosphate dehydrogenase; and 20µM hemin. The volume was made up to 400µl in buffer G. The mixture was then incubated in the dark at 37°C for one hour. The reaction was terminated by adding 400µl of chloroform and vortexing for 20 seconds, followed by centrifugation at 10,000g for one minute. The chloroform phase was then collected, and the bilirubin content measured by spectrophotometry, using the difference in absorption at 464 and 530nm, and an absorption coefficient of 40mM⁻¹cm⁻¹. Results were expressed as pmoles of bilirubin formed per mg protein per hour.

2.17 DNA Mobility Shift Assay

DNA mobility shift assays (EMSAs) were performed using two different methods – either digoxigenin or γ -³²P labelled probes. Digoxigenin labelled EMSAs were performed using a proprietary kit (DIG Gel Shift Kit, Roche) as per the manufacturer's instructions. Radioactive EMSAs were performed as previously published (Patel 2004). Oligonucleotides containing the desired consensus binding site were purchased from a commercial company (TAGN, Newcastle, UK. see appendix B for sequences). The single stranded oligonucleotides were annealed by placing 1ng of each strand in a volume of 10µl of 0.2M NaCl, and heating to 95°C

for 3 minutes, then allowing it to cool to room temperature. Briefly, the double stranded probe was labelled with γ -³²P ATP and purified on Sephadex G-25 (Pharmacia, UK) spin chromatography columns. Nuclear protein extracts were incubated in a reaction mixture with 1 μ l γ -³²P-labelled probe at 20°C for 15 minutes. Specificity of binding was ascertained by co-incubating samples with unlabelled wild type or mutant probe or an anti-HSF-1 antibody (StressGen). Protein-nucleic acid complexes were resolved using a 5% non-denaturing acrylamide gel run in 5mM Tris, pH 8.3 for 3 hours at 200V. Gels were transferred to blotting paper, dried under a vacuum at 80°C for 1 hour and exposed to radiographic film at -70°C with an intensifying screen.

2.18 Glutaraldehyde Crosslinking

Following treatment, nuclear and cytoplasmic cell extracts were obtained as described above. 20 μ g of protein was incubated with equivalent volumes of either distilled water or glutaraldehyde in 3 different doses (0, 0.02, 0.2 and 1mM respectively) for 15 minutes. The reaction was stopped by adding lysine and then the samples were boiled at 95°C for 5mins. Loading buffer was added and the samples run on a 5% polyacrylamide gel and probed for HSF-1.

2.19 Measurement of Reactive Oxygen Species

ROS generation was measured using two methods – H₂DCFDA and dihydroethidium. Both of these dyes fluoresce following exposure to ROS. H₂DCFDA is more sensitive for hydrogen peroxide and hydroxyl radicals, whilst dihydroethidium is more sensitive for superoxide, although neither is species specific (Lee 1998, Zhang 2001, Zhao 2003). For the H₂DCFDA, cells were plated in 96 well plates at a density of 2×10^4 cells per well and left overnight. The next day the cells were washed three times with HBSS and then treated as required, in the presence of H₂DCFDA at a final concentration of 10 μ M. The plate was placed in a preheated fluorimeter (at 37°C) and continuous measurement performed for 1 hour, at excitation 480nm and emission 530nm. Following this, the supernatant was removed and a protein assay was performed on the cells. The plate was snap frozen, and the protein retrieved by applying hot (95°C) Laemmli buffer. The protein concentration was measured using a proprietary kit (BioRad). The final results were corrected for variations in the protein concentration between wells and were expressed as a percentage with activity in untreated cells being 100%. Dihydroethidium was used with cells prepared as above. The required treatments were applied in the presence of 100 μ M dihydroethidium (final concentration). The cells were then placed back into normal conditions for 4 hours. The wells were washed 3 times with HBSS and ethidium fluorescence was measured with an excitation of 480nm and an emission of 560nm. Protein was retrieved from the wells as above, and assayed, allowing the final results to be corrected for protein concentration. The results were expressed as a percentage as above.

2.20 Mitochondrial Membrane Potential – DiOC6

The mitochondrial membrane potential was measured by two different techniques - 3,3'-dihexyloxacarbocyanine (DiOC6) and MitoTracker Green and Red. For the DiOC6, HUH7 cells were treated with the required dose of curcumin or vehicle for one hour, then trypsinised as described above, washed three times in PBS and resuspended in LP3 tubes in PBS at 10^6 cells/ml. They were then incubated in DiOC6 (50nM final concentration) for 15 minutes at room temperature. Propidium iodide (5 μ g/ml final concentration) was added and the cells were read immediately on an Epics XL-MCL flow cytometer (Coulter).

2.21 Mitochondrial Membrane Potential – MitoTracker

Green/MitoTracker Red

The DiOC6 method does not allow for variations in total mitochondrial mass. For this reason, a normalised mitochondrial membrane potential was measured using MitoTracker Green and MitoTracker Red. Cells were treated with the required dose of curcumin or vehicle for 1 hour, trypsinised, washed three times in PBS and then resuspended in LP3 tubes in PBS at 10^6 cells/ml. The 2 MitoTracker dyes were added at a final concentration of 100nM each, and the cells incubated at 37°C in the dark for 30 minutes. The cells were then placed on ice and read immediately on the flow cytometer. The ratio of red:green fluorescence represents the normalised mitochondrial membrane potential.

2.22 Measurement of Caspase Activity

Caspase activity was measured following curcumin treatment using the Apo-One Homogenous Caspase 3/7 Assay (Promega). This is a fluorometric method, and was performed using the manufacturer's protocol. The cells to be tested were cultured and treated as required in 96 well plates in standard conditions. Following the requisite treatment, the plate was removed from the incubator and a 1:1 ratio of Apo-One reagent was added to each well. This was mixed on a plate shaker for 30 seconds, and then the plate was incubated at room temperature for 4 hours prior to the fluorescence being read at an excitation wavelength of 485nm and an emission wavelength of 530nm. Blank and untreated control wells were performed on each occasion. 6 wells were included in each treatment group to allow statistical comparison.

2.23 siRNA Mediated Inhibition of Gene Expression

An siRNA method was used to inhibit the expression of Nrf2 and HIF-1 α , using sequences and reagents from Santa Cruz. The original manufacturer's protocol was followed. A new protocol has since been published, but it was the original method that was used for all of these experiments. 2×10^5 HUH7 cells were plated into 6well plates, in 2mls of antibiotic free medium (with 10% FCS) and cultured for 24 hours. For each well to be transfected, 6 μ l of siRNA was added to 100 μ l of Transfection medium and incubated at room temperature for 5 minutes (part A). 6 μ l of

Transfection Reagent was added to 24.2µl of Transfection medium and incubated for 5 minutes at room temperature (part B). Parts A and B were then mixed together and incubated at room temperature for 20 minutes. The cells were changed to fresh antibiotic free medium, with 1ml per well. 130µl of the siRNA mix was then added to each well. The cells were then cultured for 24 hours prior to experimentation. Controls without any siRNA, and with a control siRNA sequence were used on each occasion.

2.24 Phosphatase Activity

Phosphatase activity was measured using two proprietary kits – the Tyrosine Phosphatase Assay System and the Serine/Threonine Phosphatase Assay System, both from Promega. This is a non-radioactive molybdate-dye based kit which measures freed phosphate generated from a substrate peptide as an index of phosphatase activity. The cells were treated with curcumin for the required time. Subsequently, they were placed on ice, the supernatant removed, and the cells scraped in Phosphatase Storage Buffer (PSB), then passed through a 21G needle. The samples were centrifuged at 100,000g at 4°C for one hour. During this time the spin columns were prepared by washing with de-ionised water, and then loading with Sephadex G-25 (initially stored in Sephadex Storage Buffer) and washing with cold PSB. The columns were allowed to drain by gravity, then centrifuged at 600g for 5 minutes at 4°C to remove the remaining buffer. 250µl of the previously centrifuged supernatant is then placed on the column and centrifuged at 600g for 5 minutes at

4°C. The sample lysate is then collected from below the column and a protein assay performed as described above.

Phosphate standards are made from 0 to 2000pmol, by diluting the supplied standard (1mM KH_2PO_4) in phosphate free water, and a standard curve was generated by adding molybdate dye solution and proceeding as below. Assays were run in the supplied 96 well plates. The reaction conditions were slightly different for each of the phosphatases studied, and were varied by using slightly different buffer composition as detailed in the buffers section. The phosphopeptide used was END(pY)INASL for tyrosine phosphatase activity and RRA(pT)VA for serine/threonine activity. Premixed solutions containing 10 μl phosphatase 5x reaction buffer, 5 μl 1mM phosphopeptide and 25 μl of water were pipetted into each well. The plate was warmed to 37°C for 5 minutes. Reactions were commenced with the addition of the samples, containing 5 μg of protein in 10 μl (final reaction volume 50 μl), and the plate was incubated at 37°C for 30 minutes. The reaction was terminated by the addition of 50 μl molybdate dye solution and the colour was developed for 30 minutes at room temperature prior to being read at 630nm on a plate reader. All samples were run in duplicate. The amount of phosphate released was then calculated from the standard curve.

2.25 Enzyme Linked Immunosorbent Assays for Acute Phase

Proteins

ELISAs were performed for CRP, transferrin and fibrinogen. A standardised protocol from DAKO was used for all assays, utilising a sandwich technique. 96 well plates (Corning) were coated with a specific antibody by diluting the antibody in coating buffer at 100µl per well, and incubating it overnight at 4°C. The following morning the coating buffer was removed and the wells washed three times with wash buffer using an automated plate washer machine (Skan Washer 300, Skatron Instruments). A standard curve was prepared using commercially available standards diluted in wash buffer. In addition to the standards, wash buffer was used as a blank and medium containing 10% FCS was used as a further control. Supernatants were prepared for addition to the plate. Those from cell lines were used neat, whilst those from cultures of primary hepatocytes were diluted 1 in 5 in wash buffer. 100µl of test sample or standard was added to each well, and all samples were assayed in duplicate. Quality controls of a known concentration were placed on the plate near the beginning and end to provide assessment of intra-assay variability. The plate was then incubated at room temperature for two hours. Subsequently, the plate was washed as above, and 100µl of an HRP conjugated primary antibody (different to the coating antibody) was added and the plate incubated for one hour at room temperature.

The plate was then washed again and 100µl of chromogenic substrate (DAKO OPD tablets, diluted as per the manufacturer's instructions) was added to each well. The

plate was covered, and placed in the dark at room temperature to develop for fifteen minutes. Subsequently the development was stopped by adding 100µl of 0.5M sulphuric acid to each well. The results were then read using a Dynatech plate reader (Dynex MRX II, Dynatech) on a 490nm filter (reference filter 630nm). A standard curve was obtained from the plate reader, using a semilog fit for the known standards. This was then used to calculate the concentrations of the assayed samples.

2.26 Cytochrome p450 Assays

Cytochrome p450 activity in primary cultures of human hepatocytes following HO-1 induction was measured using a luminescent kit from Promega – the P450-Glo Assay System. The assay was performed according to the manufacturer's protocol. Three different isoforms were tested – 1A2, 2C9, and 3A4. These all require slightly different conditions and buffers. The optimal conditions for each isoform are detailed in appendix B. The general protocol follows. 12.5µl of 4x substrate reaction mix and 12.5µl of cell lysate was added to each well of a 96 well plate. 25µl of 2x NADPH generating system was added, and the reaction was incubated as required. 50µl of luciferin detection reagent was then added, the reaction was incubated at room temperature for 20 minutes and the plate was read in a luminometer. The amount of protein in each cell lysate was measured using the protein assay described earlier, and the results were corrected for the amount of protein, and then expressed as a percentage of untreated controls.

2.27 Urea Assay

Measurement of urea production was performed using an adaptation of a commercially available Urea Nitrogen Kit (Sigma), modified to allow analysis in 96 well plates. Cell culture supernatants were used for analysis, and all samples were performed in triplicate. 25µl of sample was placed in each well, and 50µl of urease solution was added, and the plate incubated at 37°C for one hour. Subsequently, 25µl each of phenol nitroprusside and alkaline hypochlorite solution were added to each well, in addition to 125µl of distilled water. The plate was briefly agitated and incubated at room temperature for thirty minutes before being read at 630nm on a plate reader. To allow quantitative analysis and comparison of results between plates, a standard curve assayed on each plate, using a urea standard diluted to provide a 0 to 500µM range.

2.28 ATP Assay

ATP levels in hepatocytes following treatment was performed using the ATP Assay Kit from Calbiochem (catalogue number 119107) in accordance with the manufacturer's instructions. This method uses luciferin and luciferase, and the amount of luminescence generated indicates the amount of ATP present within the sample. The medium was removed from the treated cells (20,000 per well in 96 well plates), which were then treated with 80µl Nucleotide Releasing Reagent (NRR) for 5 minutes with shaking at room temperature. 20µl of further NRR containing 1µl of

ATP monitoring enzyme was then added and the sample was read immediately in a luminometer as described for the luciferase reporter assay. Negative controls containing no cells and untreated controls were assayed on all runs.

2.29 Data Analysis

All values are expressed as means \pm standard error (SEM). SPSS software was used for statistical analysis (SPSS, IL, USA). Excel (Microsoft) software was used for simple descriptive statistics. The majority of data was non-parametric, and so Wilcoxon's Sum Rank test was used for statistical analysis. The differences between groups were considered to be significant at $P < 0.05$.

Chapter 3

Curcumin and the Stress Response

3.1 Introduction

Curcumin has been shown in previous studies to induce several stress proteins. The aim of this chapter was to explore the pattern of stress protein induction by curcumin in human hepatocytes.

Curcumin is a derivative from the root of the plant *Curcuma longa*, and is the major constituent of the spice turmeric. It has a wide variety of biological effects and has long been used in Chinese and Indian medicine for its medicinal properties, which are predominantly anti-inflammatory in nature (Ammon 1991). It is for this reason, with the pressure to develop new therapies for medical use, that there has been a significant interest in the mechanisms through which curcumin may work to exert its effects.

At a cellular level, curcumin has been shown to have a variety of effects on the expression of stress proteins. It has been shown to induce Hsp70 in HeLa cells (Dunsmore 2001), through activation of HSF-1. It can also induce HO-1 (Motterlini 2000). Curcumin has also been described as having a potentiating effect on cells undergoing stress, enabling higher expression of heat shock proteins such as Hsp70 and Hsp27 in response to a set stimulus (Kato 1998). The effect of curcumin on human hepatocytes has not previously been examined.

Aim: To establish the profile of stress protein expression in human hepatocytes following curcumin treatment.

3.2 Results

The pattern of stress protein expression was studied using the methods described in chapter 2, with mRNA and protein levels and reporter activity used as outcomes.

These studies necessitated preliminary dose toxicity and vehicle experiments.

3.2.1 Choice of vehicle

Curcumin is highly lipophilic and was found to be virtually insoluble in water or cell culture medium. It was found to be soluble in both ethanol and dimethylsulfoxide (DMSO) at maximum concentrations of 10 and 100mM respectively. Initial experiments suggested that the use of DMSO would minimise the contributing effect of any vehicle to stress protein induction, as ethanol was found to mildly induce Hsp70 in the required dose range. The highest concentration used was 100 μ M, which resulted in a 1% (v/v) concentration of ethanol. Thus DMSO was used for all subsequent experiments with curcumin.

3.2.2 Stress protein expression

The pattern of heat shock protein expression in response to curcumin treatment was examined in HUH7 cells. Initial experiments were guided from the literature, and doses ranging from 100 μ M to 1nM were used. Cells were treated with curcumin and western blots were run for Hsp70 initially as a protein that was expected to be induced. There was no response in Hsp70 in the range tested, despite the dose

response being repeated using a variety of treatment durations. Subsequent western blots were performed for other Hsps including HO-1 which was found to be induced at 10 μ M. Having identified an approximate target dose, all subsequent experiments were performed using a dose range from 1 to 50 μ M.

Maximal induction of HO-1 was confirmed to be after treatment with 10-25 μ M curcumin (figure 3.1). Time course experiments revealed that induction of HO-1 occurred after treatment of between 4 and 12 hours (figure 3.2). Induction of HO-1 occurred at 6 hours after initial exposure to curcumin, even if the treatment had only lasted one hour. Further western blots performed on cells treated with curcumin at the above doses and times demonstrated that there was no induction of Hsp70, BiP (Grp78), Grp94, Hsp90, or Hsp27 (figure 3.1). Identical results were found when the above experiments were repeated in primary cultures of isolated human hepatocytes.

3.2.3 Localisation of HO-1 within the liver

Fresh slices of human liver were cultured as described in chapter 2, in the presence of 10 μ M curcumin or controls for 6 hours. These were then fixed in formalin, slides cut and immunohistochemistry performed for HO-1 as described. There was evident concentration of HO-1 expression within Kupffer cells in untreated liver, with a low basal level of HO-1 expressed in hepatocytes. The localisation of HO-1 in Kupffer cells was unchanged following curcumin treatment, and there was increased staining in the hepatocytes, consistent with the western blot results in isolated hepatocytes. This effect was not seen with vehicle controls (figure 3.3).

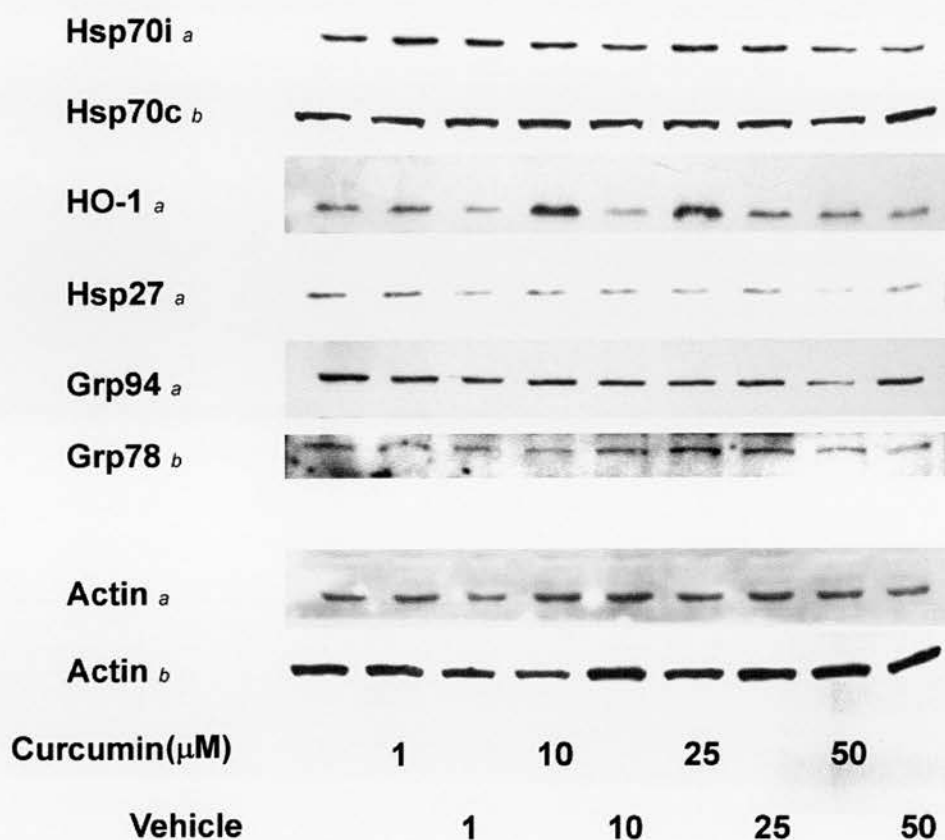


Figure 3.1 Curcumin induces HO-1 but not Hsp70 in HUH7 cells.

Western blot of whole cell lysates from cells treated with curcumin for 8 hours run in parallel with matched vehicle controls as indicated. HO-1 was induced after treatment with 10 and 25 μ M curcumin compared with vehicle and nil controls. No other consistent increases were seen. Grp 94 and Hsp27 were reduced after 50 μ M curcumin, reflecting toxicity at this dose. Blots shown are representative of three separate repeats. Results shown are from two different membranes *a* and *b*. Both actin loading controls are shown and the corresponding membranes indicated adjacent to each strip.

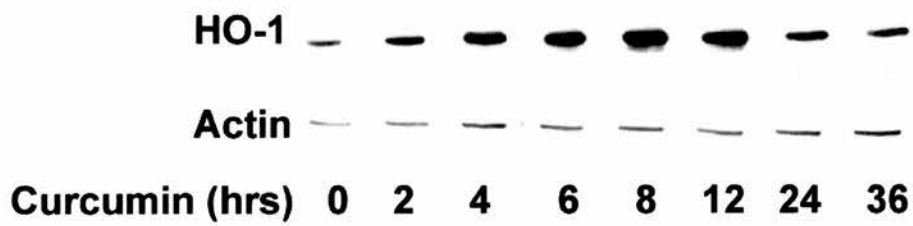
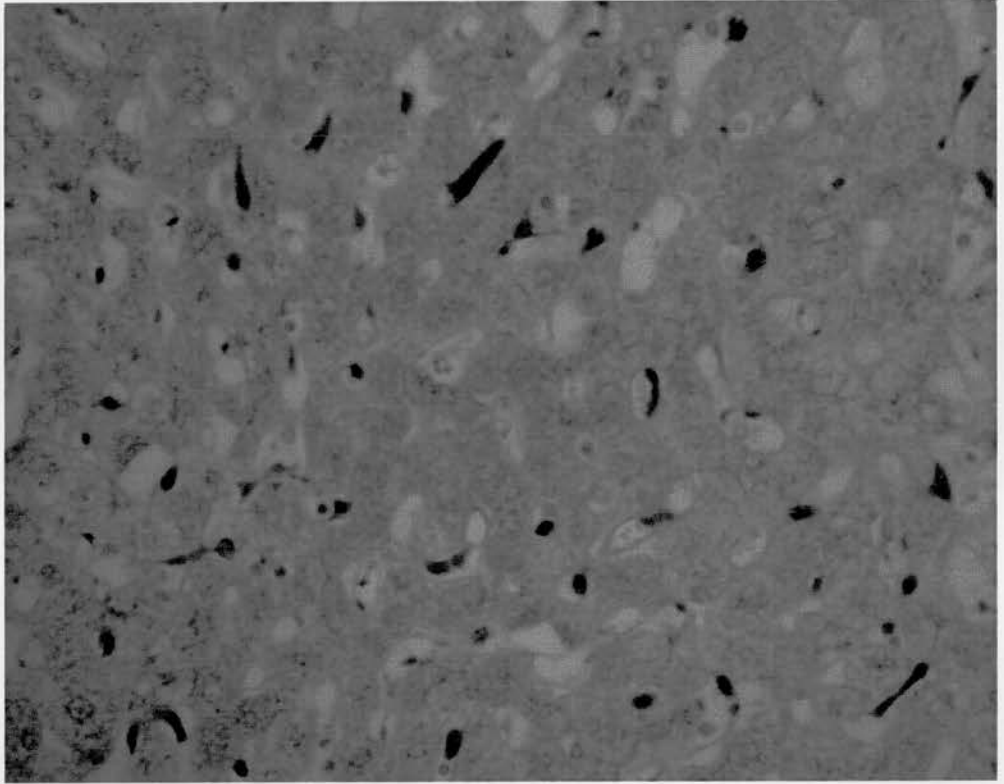


Figure 3.2 Curcumin induces HO-1 in a time dependent manner.

HUH7 cells were treated with 10 μ M curcumin for increasing periods of time up to 36 hours and HO-1 expression measured by western blot. Maximal induction of HO-1 was seen between 4 and 12 hours of treatment.

A



B

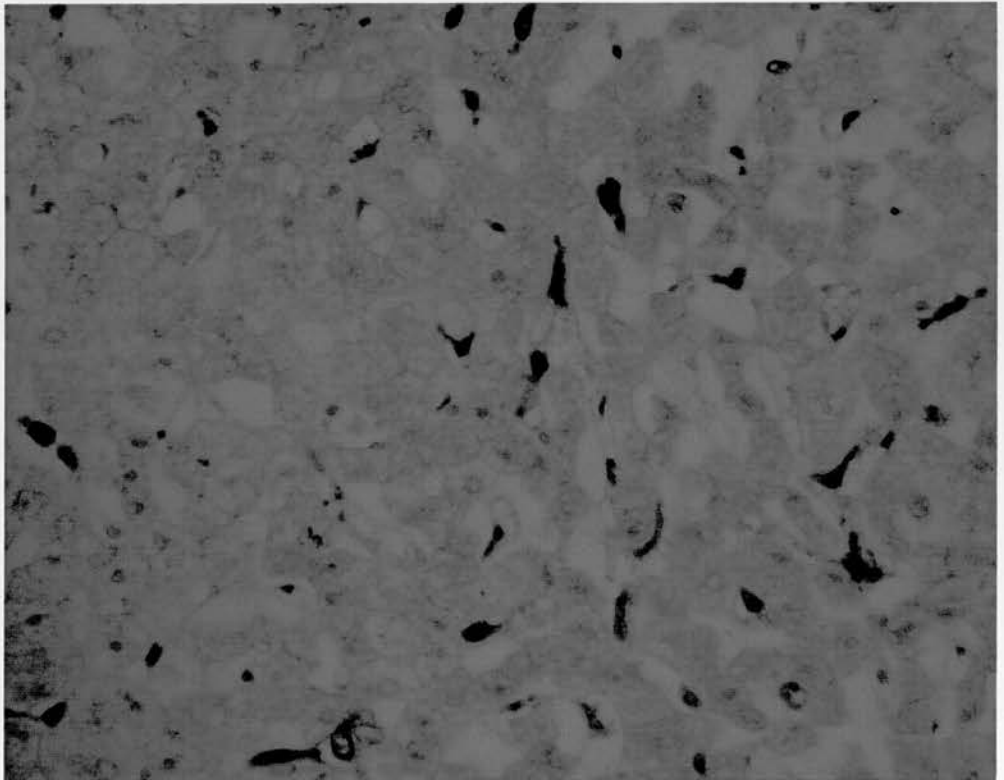


Figure 3.3 Immunohistochemistry for HO-1 in Cultured Liver Slices.

A. Treatment with 10uM curcumin for 6 hours. **B.** Equivalent DMSO for 6 hours.

3.2.4 Curcumin demonstrates dose dependent toxicity

HUH7 cells and isolated human hepatocytes were treated with curcumin or matched vehicle controls for 24 hours, in doses ranging from 1 to 100 μ M curcumin. Survival was then assessed by MTT. Both cell types responded in a similar manner, with doses of 100 μ M being uniformly toxic and doses of 10 μ M being well tolerated with viability virtually 100% of controls. There was a clear relationship of dose to toxicity in treatments between 10 and 100 μ M (figure 3.4).

HUH7 cells were treated with increasing doses of curcumin and the degree of caspase 3/7 activity was measured. Caspase activity was increased after treatment with curcumin at 50 μ M or greater ($p < 0.05$, figure 3.5). A similar induction of caspase activity was seen whether it was measured after 6 or 24 hours of curcumin treatment.

3.2.5 Confirmation of HO-1 induction

Initial results with western blotting had demonstrated that HO-1 protein was induced by 10 μ M curcumin. In order to confirm this induction was the result of *de novo* synthesis and led to an increase in functional enzymatic activity of HO-1, further experiments were performed to measure HO-1 promoter activity using a human HO-1 reporter, measure changes in mRNA levels, and assess heme oxygenase activity after 8 hours of treatment with 10 μ M curcumin.

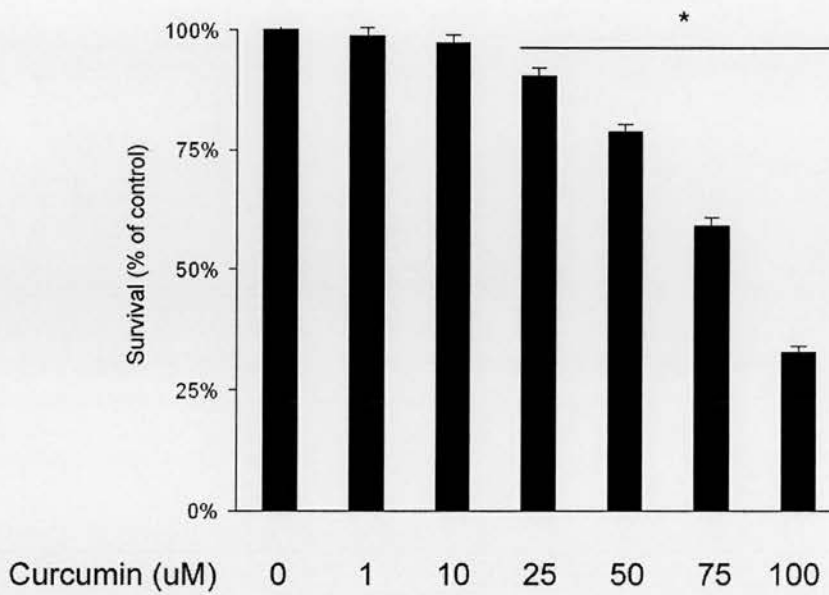
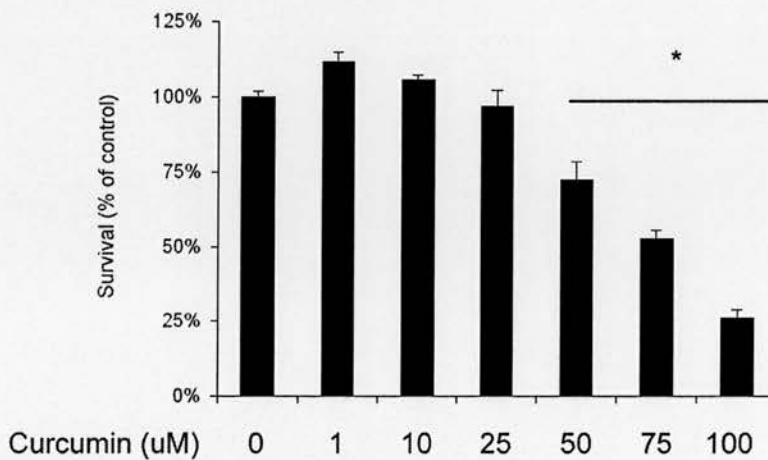
A**B**

Figure 3.4 Curcumin demonstrates dose dependent toxicity.

MTT assay demonstrating dose dependent curcumin mediated toxicity.

A. HUH7 cells. **B.** Primary human hepatocytes. Cells were treated for 24 hours, before cell viability was assessed. Data are expressed as a percentage of the medium control, and represent the mean \pm SEM of 6 wells. * $p < 0.01$.

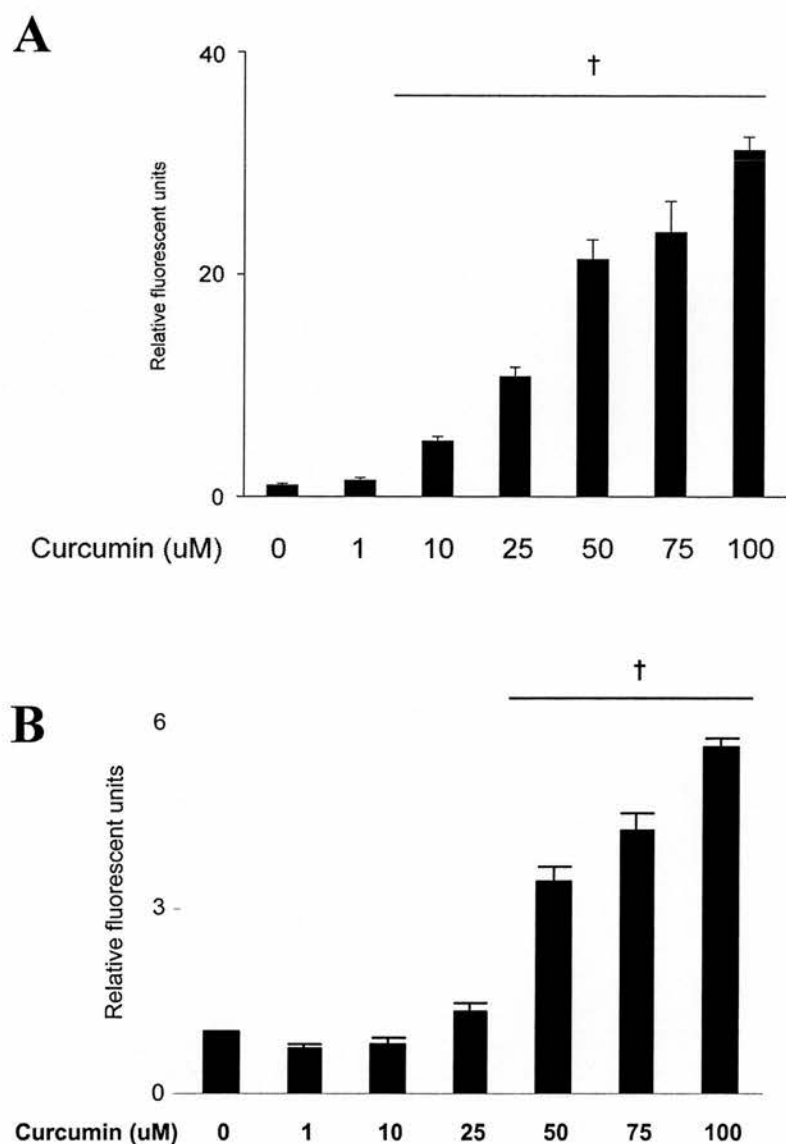


Figure 3.5 Curcumin treatment activates caspase 3/7.

HUH7 cells were treated with curcumin for 6 or 24 hours (panels A and B respectively) and caspase 3/7 activity measured. Caspase activity was increased at doses of 50 μ M and above ($\dagger = p < 0.05$ vs nil control). Each bar represents the mean \pm SEM of six repeats from one experiment. Each experiment was performed 3 times with similar results.

HO-1 reporter activity was assessed in HUH7 cells. Activity was significantly increased with curcumin treatment (10 μ M for 8 hours) with a mean induction of 1.75 fold greater than controls (* p <0.01, figure 3.6). Vehicle had no effect on reporter activity.

HO-1 mRNA was measured using real time PCR which was performed on both HUH7 cells and on isolated human hepatocytes. HO-1 mRNA levels were significantly increased by curcumin treatment in both cell types (\dagger = p <0.05, figure 3.7). The results obtained in HUH7 cells were very consistent, with a mean induction of 535% of control cells. Whilst HO-1 mRNA was always increased by curcumin treatment, there was much greater variability in the results obtained with isolated hepatocytes, with a range of induction from 2 to 2000 fold, and a median induction of 6.5 fold over untreated controls.

Heme oxygenase activity was measured in HUH7 cells following curcumin treatment. Total HO activity was increased approximately 2.5 fold from 171 to 455 pmoles bilirubin/mg protein/hour (* p <0.01, figure 3.8). Vehicle had no significant effect, lowering the activity slightly to 148 pmoles bilirubin/mg protein/hour.

In order to assess the effectiveness of curcumin in inducing HO-1, it was compared with the HO-1 inducing ability of equimolar amounts of the classical HO-1 inducer hemin. It was found that curcumin induced HO-1 to a similar degree and that there was no evidence of synergy between the two inducers when given together (figure 3.9).

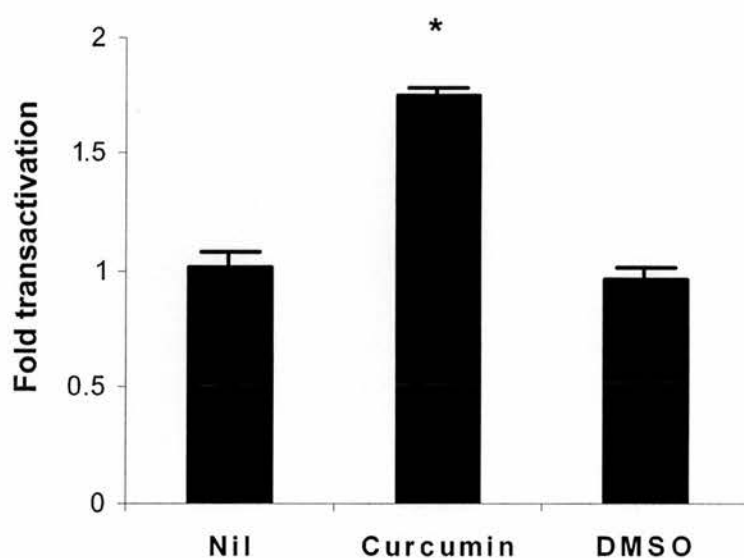


Figure 3.6 Curcumin treatment increases HO-1 reporter activity.

HUH7 cells were co-transfected with a human HO-1 reporter and a control vector. HO-1 activity was elevated following treatment with 10 μ M curcumin for 5 hours. **DMSO** vehicle control. Results shown represent mean \pm SEM of 6 independent repeats.

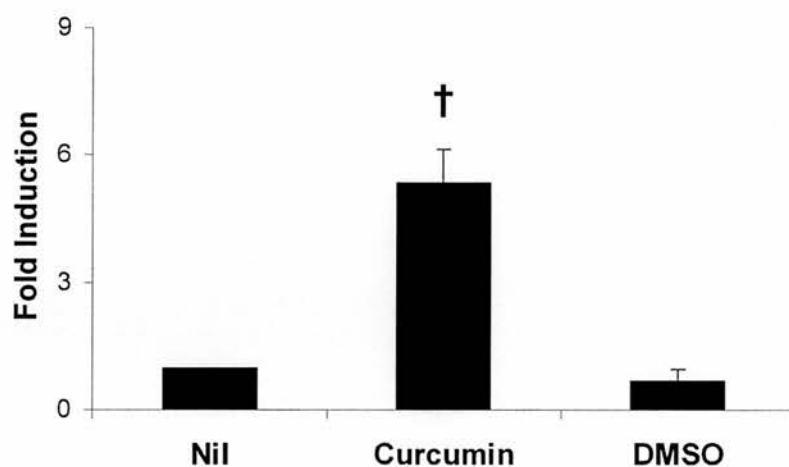
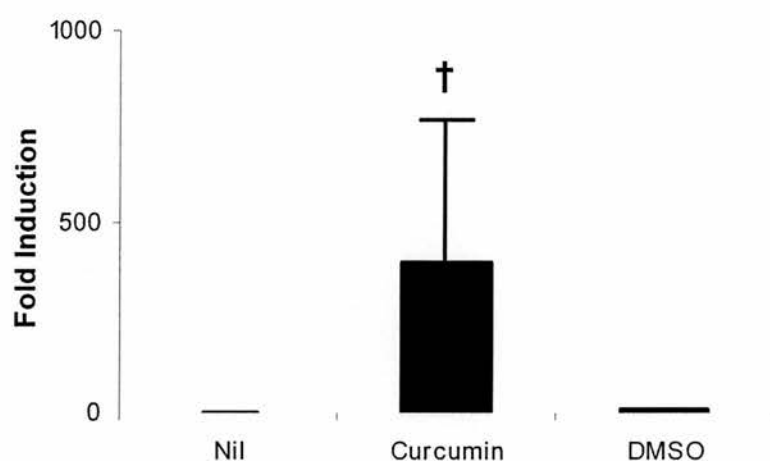
A**B**

Figure 3.7 Curcumin increases HO-1 mRNA levels in human hepatocytes.

A. HUH7 cells. **B.** Primary human hepatocytes. Cells of each type were treated with 10 μ M curcumin for 5 hours and mRNA levels measured using Real Time PCR. Data shown represent mean \pm SEM of 6 independent repeats. [†]p<0.05. **DMSO** vehicle control.

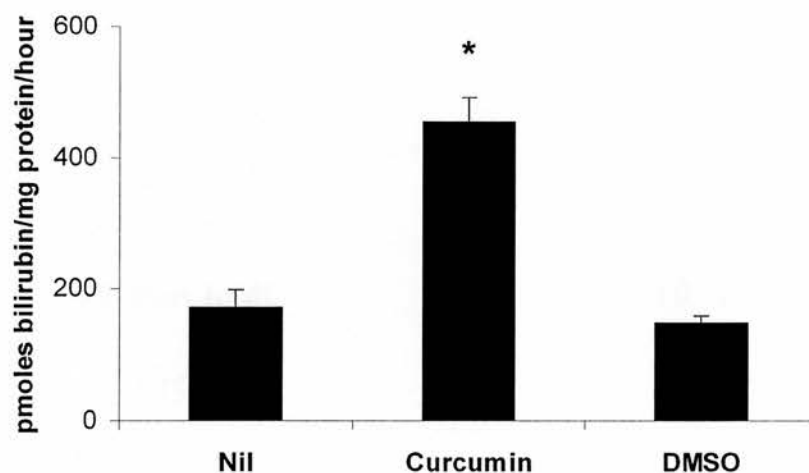
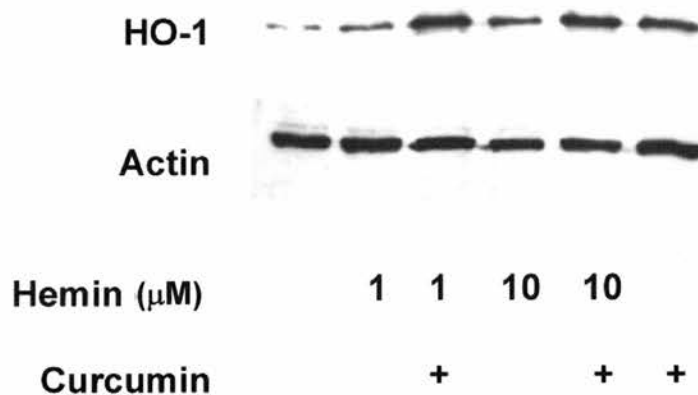


Figure 3.8 Curcumin increases HO-1 activity.

HUH7 cells were treated with 10 μ M curcumin for 6 hours and then HO-1 enzymatic activity was measured. Curcumin significantly increased HO-1 activity (* $p < 0.01$) from 171 to 415 pmoles bilirubin/mg protein/hour. Results shown are the mean \pm SEM of 6 independent repeats.

A



B

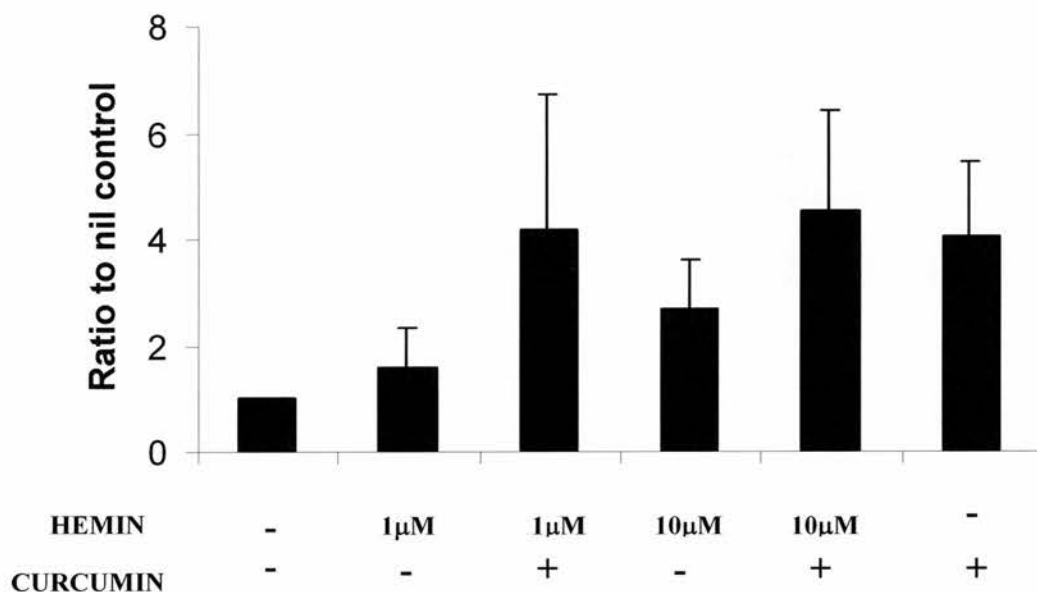


Figure 3.9 Curcumin is as effective as hemin as an inducer of HO-1.

A. Western blot of whole cell lysates from cells treated with hemin and curcumin (10 μM) showing that the addition of hemin does not increase HO-1 levels beyond that attained by curcumin alone. **B.** Quantitation by densitometric scanning of the bands. The results shown represent the means \pm S.E.M. of three independent experiments.

3.2.6 Confirmation of lack of Hsp70 induction

The western blotting results showing that curcumin did not induce Hsp70 were surprising and ran contrary to expectations based on the published literature (Dunsmore 2001, Chen 2001). In view of this, confirmation was sought using a reporter for the human Hsp70 promoter, and mRNA levels were measured with real time PCR.

HUH7 cells transfected with the Hsp70 reporter were treated with 10 μ M curcumin or vehicle for 8 hours. Curcumin and DMSO were both found to reduce the activity of the reporter significantly to 15 and 22% of untreated controls respectively (* p <0.01 vs untreated control, figure 3.10).

Hsp70 mRNA levels were measured in HUH7 cells and in primary human hepatocytes treated with 10 μ M curcumin. There was a modest 2.5 fold induction of Hsp70 mRNA in HUH7 cells (\dagger = p <0.05, figure 3.11), whilst vehicle had no effect. In primary cultures, there was induction of the Hsp70 mRNA levels to 38 fold greater than untreated controls. As seen in the HO-1 mRNA results, this was skewed by one large induction of Hsp70, although the median induction was 16 fold, with a range of 7 to 113 fold greater than untreated controls. The DMSO vehicle also had a modest effect and induced Hsp70 mRNA 4 fold (not significant). For comparison, Hsp70 mRNA was measured in response to heat shock (43°C for 45 minutes), and was found to rise dramatically with a median induction of 20 fold in HUH7 cells and 900 fold in primary human hepatocytes.

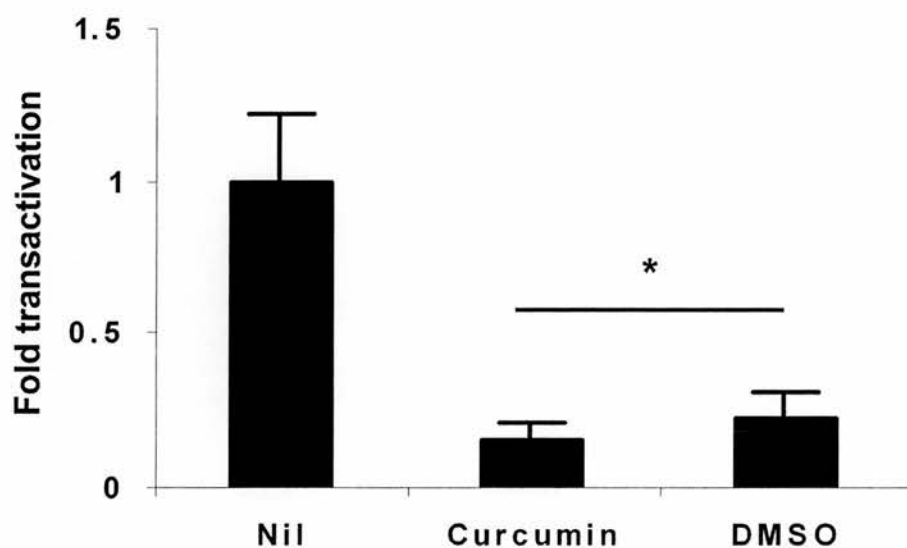


Figure 3.10 Curcumin does not induce Hsp70 reporter activity.

HUH7 cells were co-transfected with a human Hsp70 reporter and a control vector. Reporter activity was measured after 5 hours of treatment with 10 μ curcumin or equivalent vehicle control (**DMSO**). Both led to a reduction in reporter activity (* $p < 0.01$ vs nil control). There was no significant difference between treatment with curcumin and its vehicle. Data represent the mean \pm SEM of 6 repeats.

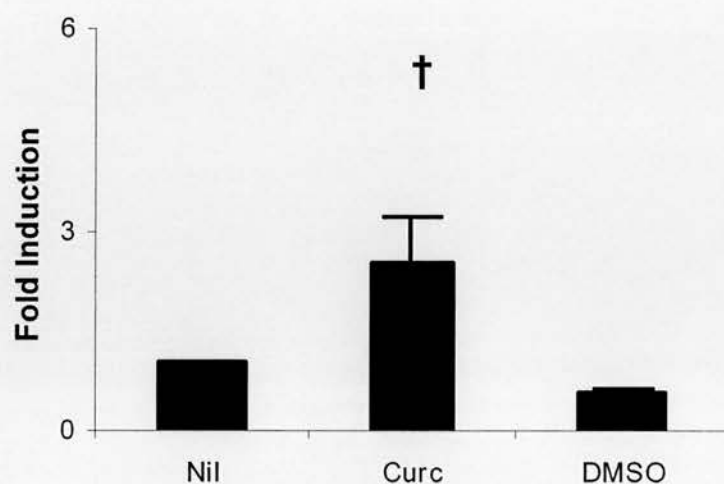
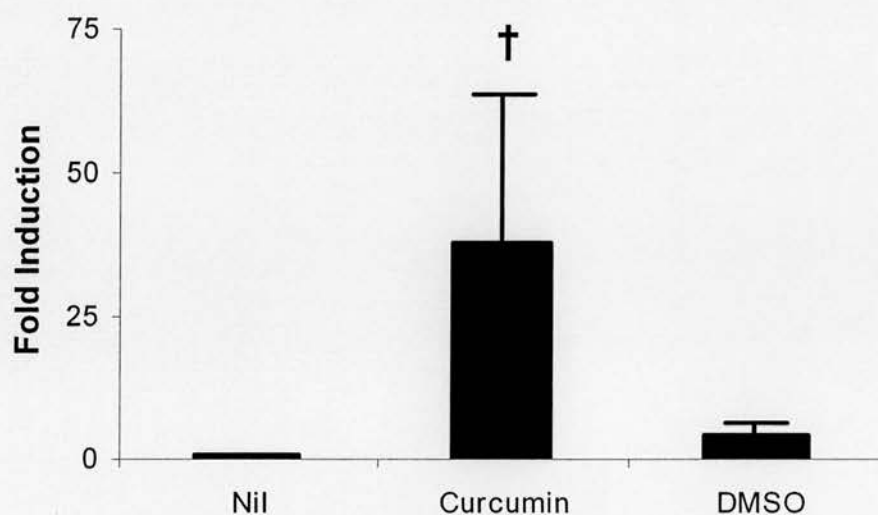
A**B**

Figure 3.11 Curcumin increases Hsp70 mRNA levels in human hepatocytes.

A. HUH7 cells. **B.** Primary human hepatocytes. Both cell types were treated with 10 μ M curcumin or equivalent vehicle (**DMSO**) for 5 hours and Hsp70 mRNA levels measured by Real Time PCR. Curcumin significantly increased Hsp70 mRNA levels in both cell types († $p < 0.05$). Data shown represent the mean \pm SEM of 6 repeats.

In light of the contradictory findings of the Hsp70 reporter and mRNA measurement, the level of Hsp70 mRNA was explored in more detail using HUH7 cells. A time course of Hsp70 mRNA following curcumin or heat shock treatment was performed, and it was demonstrated that the rise in Hsp70 mRNA with curcumin treatment was very modest in comparison to that seen with heat shock (figure 3.12A). However, this was still not consistent with the apparent lack of reporter activity. Hsp70 mRNA levels can be increased by post transcription means, and so the effect of curcumin on Hsp70 mRNA levels was assessed during treatment with the transcriptional inhibitor actinomycin D (figure 3.12B). There was a rapid reduction in the level of Hsp70 mRNA following treatment with actinomycin D to 11% of control levels in cells that received no treatment. This drop was prevented by treatment with 10 μ M curcumin, which resulted in a maintenance of Hsp70 mRNA at approximately 100% for 3 hours, although this dropped to 43% after 5 hours of treatment. Consistent with known post transcriptional mechanisms, heat shock was able to increase Hsp70 mRNA levels even in the presence of actinomycin D, which was sustained for several hours before decaying. This experiment was performed twice with similar results, although this was insufficient to permit statistical comparisons.

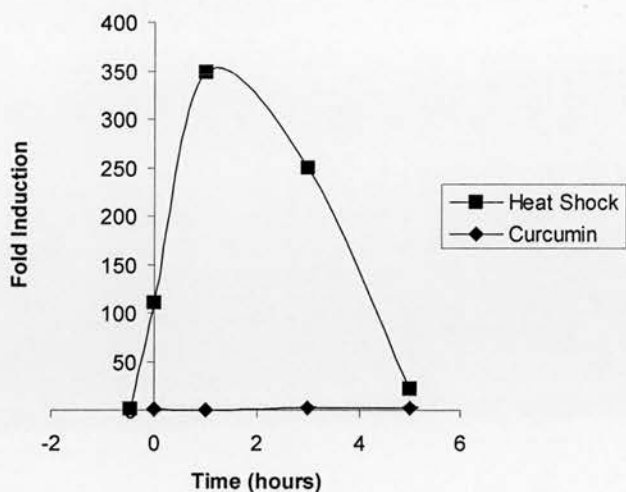
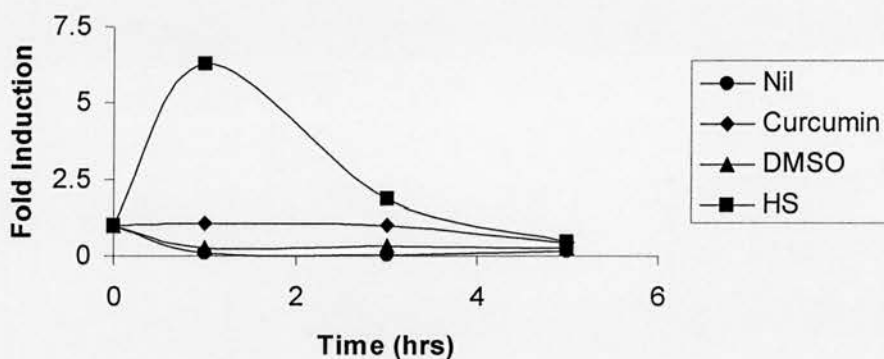
A**B**

Figure 3.12 A. Heat shock strongly induces Hsp70 mRNA.

Time course of Hsp70 mRNA induction following heat shock (43°C for 45 mins) or curcumin treatment (10 μ M). Curcumin led to a very modest induction of Hsp70 in comparison with the large (>300 fold) increase seen with heat shock.

B. Hsp70 Curcumin stabilises Hsp70 mRNA levels. HUH7 cells were treated with curcumin or vehicle (DMSO) or heat shock (HS, 43°C for 45 mins) in the presence of actinomycin D (5 μ g/ml). Curcumin treatment delayed the decline in HSp70 levels seen in control and vehicle treated cells.

3.3 Discussion

Curcumin has been described in the literature as a potent inducer of HO-1 and Hsp70 (Motterlini 2000, Dunsmore 2001) although this ability had not previously been examined in human liver cells. The intention of this work was to ascertain whether curcumin was able to induce this response in human hepatocytes. Curcumin induces HO-1 in bovine endothelial cells and in renal epithelial cells (Motterlini 2000, Balogun 2003). However, the human HO-1 promoter differs significantly to that of other species, and certain stimuli which induce HO-1 in rodent cells either repress HO-1 or have no effect in human cells (Sikorski 2004). For this reason it could not be assumed that curcumin would induce HO-1 in human hepatocytes, and this required evaluation. The induction of HO-1 was confirmed by human hepatocytes, both in the HUH7 cell line and in primary cultures of isolated human hepatocytes. There was a consistent induction in all lines of investigation which resulted in a clear increase in functional activity.

In contrast, there was no identifiable elevation in Hsp70 levels with curcumin treatment. There are several potential reasons for this result. Hsp70 induction is one of the cell's responses to injury, and previous reports of Hsp70 induction by curcumin have been associated with impairment of cell survival. Curcumin was found to be toxic when administered in doses greater than 25 μ M. As discussed in the introduction (see section 1.6.1.2), curcumin has been found to have either pro-oxidant or anti-oxidant properties, dependent upon cell type and conditions (Piwocka 2001, Khar 2001, Ghoneim 2002, Woo 2003, Ligeret 2004). It is likely that the toxic

profile demonstrated here reflects its pro-oxidant properties in inducing reactive oxygen species generation (chapter 5). The results are consistent with the toxicity seen in other groups work (Dunsmore 2001). However, even when administered at toxic doses, there was still no rise in Hsp70 protein. This may be because the mechanism of curcumin- induced toxicity in our cells does not permit the induction of Hsp70. It has been established that curcumin has different effects in different cell lines, and that some do not respond by producing Hsp70. Indeed, Khar found that those cells most sensitive to the pro-apoptotic effects of high dose curcumin produced no Hsp70 response (Khar 2001). It is likely that this was the case in the HUH7 cells.

The mechanism of cell death which curcumin triggers has been a matter of debate, with conflicting reports being published regarding caspase activation and a classical mitochondrial pathway, and non-classical pathways in different cell types (Anto 2002, Piwocka 1999). In HUH7 cells there was clear induction of caspase 3/7 activity consistent with findings in hepatocyte, renal and leukaemia cell lines (Gomez-Lechon 2002, Woo 2003, Anto 2002). Caspase 3 is the common end-mechanism through which caspase dependent apoptosis occurs. However, this was the only method used to assess the mechanism of cell death in relation to curcumin toxicity, and as such, it cannot be confirmed that definite apoptosis of the cells occurred, only that there was an increase in caspase activity which is consistent with apoptosis. It is traditionally thought that apoptosis and necrosis are distinct pathways to cell death, however, Jaeschke has described an indeterminate mechanism termed necrapoptosis, the occurrence of which is dependent on the energy state of the cell

(Jaeschke 2003). It is possible that this is the mechanism of cell death in some of the curcumin studies where caspase activation has not been found to occur. Indeed, the finding of lower caspase activity in cells that were treated with curcumin for 24 hours may reflect necrosis or necrapoptosis.

Whilst Hsp70 protein was not elevated by curcumin treatment, there were conflicting results regarding the Hsp70 reporter and mRNA levels. Reporter activity was reduced, suggesting that there was a reduction in activity of the Hsp70 promoter. However, mRNA levels were increased. The reporter used in this study is a commercially available Hsp70 reporter containing 2.5kb of the human Hsp70B promoter (Stressgen). It would be expected that this would be of sufficient length to encompass most of the functional upstream units in the promoter, and it has been used successfully by many groups (Cippitelli 2003, Suzuki 1998), although far removed enhancer regions will not be included. It does contain the HSF-1 binding motif which is central to the heat responsive character of the protein, and therefore if curcumin were inducing HSF-1 activity, it would be expected to respond. The lack of response is consistent with the lack of HSF-1 activity following curcumin treatment (chapter 4).

The reporter is β -galactosidase based and the method of detection may not be sufficiently sensitive to monitor subtle changes in reporter activity. Even though major induction seen in response to heat shock can be detected, the response to heat is of a different magnitude, as demonstrated by the mRNA results. Thus minor induction of Hsp70 promoter activity may be missed due to the assay conditions.

However, even if there was a subtle induction of Hsp70 promoter activity resulting in increased mRNA levels, it was insufficient to produce a demonstrable increase in Hsp70 protein as measured by western blot.

Hsp70 mRNA levels were increased both in HUH7 cells and in primary cultures of isolated hepatocytes. There was a marked difference in the level of induction seen in the two cell types, and the primary cultures showed a wider spread of level of induction. This is the result of many factors such as the differences between the individuals from which the cells were isolated, in comparison to the controlled environment of the HUH7 cells which would be expected to give more consistent results. Indeed, the variability between hepatocyte preparations was also observed in HO-1 mRNA levels.

Hsp70 mRNA levels were consistently elevated after curcumin treatment. The level of induction was very low in comparison to that seen in response to heat shock. The reason for this comparison was the suspicion that the level of mRNA achieved may not produce detectable increases in Hsp70 protein. Heat shock represents a standard inducer which does produce detectable increases in Hsp70 protein. There is a great redundancy in Hsp70 mRNA levels (Theodorakis 1987) and a modest induction of Hsp70 mRNA may not result in an increase in translation and hence no change in protein levels. Hsp70 mRNA also has many post transcriptional controls, and it can be stabilised following physical or chemical stress (Theodorakis 1987, Kaarniranta 1998, Alfieri 2002). The results of the experiments with actinomycin D suggest that curcumin may stabilise Hsp70 mRNA, and this stabilisation may be the reason for

the increase in Hsp70 mRNA levels in the absence of increased promoter activity. This experiment was performed twice, and so no statistical comparison can be made, although the results were similar on both occasions.

3.4 Conclusion

The above experiments demonstrate that curcumin can readily induce HO-1 in hepatocytes and that Hsp70 protein is not induced even after administration of toxic doses of curcumin. However, curcumin did consistently increase Hsp70 mRNA levels, and this remained incompletely explained. Hsp70 is induced by the transcription factor heat shock factor 1 (HSF-1), which has been shown in other cell lines to be activated by curcumin treatment (Dunsmore 2001). The human HO-1 promoter also contains an HSF-1 consensus binding site. This raised the question of what effect curcumin treatment had on HSF-1.

Chapter 4

Curcumin and Heat Shock Factor 1

4.1 Introduction

Curcumin is reported in the literature to activate the transcription factor heat shock factor 1 (HSF-1) (Dunsmore 2001, Chen 2001). In human hepatocytes curcumin was found to induce HO-1 and increase Hsp70 mRNA levels (see chapter 3), both of which could result from increased HSF-1 activity. The aim of this chapter was to examine the effect of curcumin treatment on HSF-1 activation in human hepatocytes.

Heat shock factor 1 is the main transcription factor regulating the heat responsive nature of heat shock proteins (Morimoto 1992). It has a complex structure and activation process. In resting cells it exists in an inactive monomeric form in the cytoplasm. Activation involves it migrating to the nucleus, and undergoing a conformational change which allows homo-trimerisation and hyperphosphorylation. It is the trimeric hyperphosphorylated form which is required for transcriptional activation (Jurivich 1995).

This complex process can be examined at each stage and certain stimuli also can cause potential activation (Jurivich 1995).

Aim: To examine the effect of curcumin administration on HSF-1 activation in human hepatocytes.

4.2 Results

4.2.1 Nuclear localisation of HSF-1

Curcumin had been shown to increase Hsp70 in a dose dependent manner in HeLa cells in the range of 10-30 μ M (Dunsmore 2001). In HUH7 cells there is a clear dose dependent toxicity after treatment with doses over 25 μ M (chapter 3), and Hsp70 mRNA had been found to rise after treatment with 10 μ M curcumin. For this reason, nuclear localisation of HSF-1 was examined using doses ranging from 10 to 50 μ M.

Curcumin treatment for two hours led to nuclear localisation after treatment with 50 μ M (figure 4.1). There was no consistent, discernible effect on HSF-1 nuclear localisation after treatment with doses of 10 and 25 μ M.

4.2.2 Activation of HSF-1

The profound nuclear localisation of HSF-1 seen following treatment with 50 μ M curcumin was likely to represent a toxic effect. In view of the desire to assess curcumin as a therapeutic inducer of the stress response, further examination of HSF-1 proceeded using non-toxic doses. 10 μ M was chosen because an increase in Hsp70 mRNA was seen with treatment at this dose (chapter 3).

Nuclear HSF-1 was examined by non-denaturing gel electrophoresis to assess whether it had trimerised. HUH7 cells were treated with 10 μ M curcumin for two

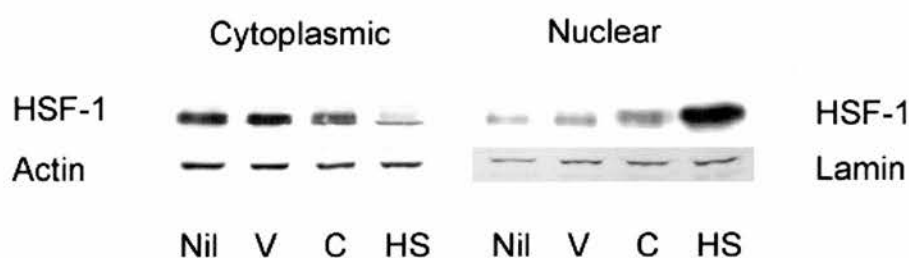


Figure 4.1 Toxic levels of curcumin lead to nuclear localisation of HSF-1.

HUH7 cells were treated with curcumin 50 μ M or vehicle for 2 hours prior to lysis. Other cells were treated solely with medium or exposed to heat shock (43°C for 45 mins). Nuclear and cytoplasmic extracts were obtained and western blotting was performed for HSF-1. **Nil**, medium only; **V**, DMSO vehicle equivalent to curcumin dose for 2 hours; **C**, curcumin 50 μ M for 2 hours; **HS**, heat shock. Actin was used as a loading control for the cytoplasmic extracts, and lamin for the nuclear extracts.

hours and native extracts obtained. The HSF-1 was found to have remained in the inactive monomeric form distinct from the trimeric form induced by heat shock (figure 4.2).

The monomeric form of HSF-1 is capable of constitutive binding to a consensus heat shock element sequence, and so this was assessed by gel mobility shift assay (EMSA). HUH7 cells were treated with 10 μ M for increasing times up to two hours. The curcumin treated extracts contained HSF-1 that bound to the consensus sequence. However this was distinct from the transcriptionally active trimeric form seen following heat shock (figure 4.3).

The final step in activation of HSF-1 is hyperphosphorylation, which is a requirement for transactivation. This was examined by relative electrophoretic mobility following in vitro treatment of cell lysates with alkaline phosphatase. The lysates were nuclear extracts from HUH7 cells which had been treated with 10 μ M curcumin for two hours. Comparison of HSF-1 from curcumin or heat shocked cells revealed that curcumin does not induce the hyperphosphorylation of HSF-1 that is seen with heat treatment. Curcumin treated cells show the same degree of constitutive phosphorylation as control cells (figure 4.4).

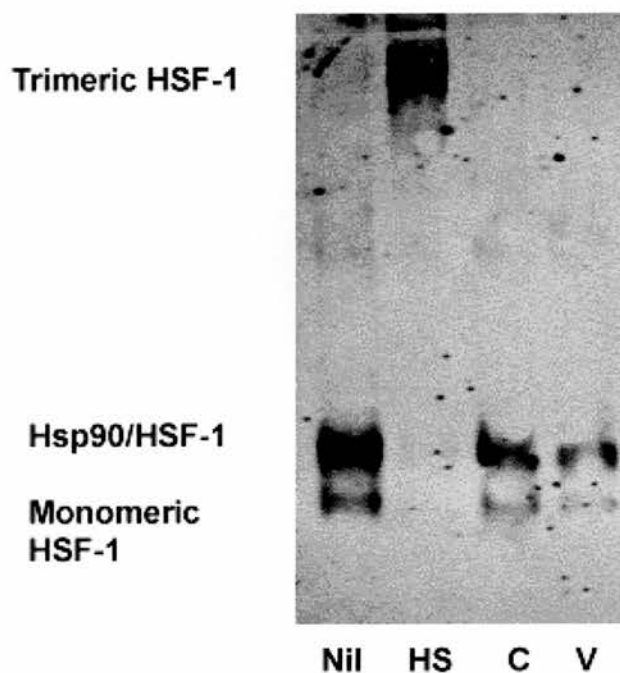


Figure 4.2 Curcumin does not activate HSF-1 at non-toxic doses.

Cells were treated with 10 μ M curcumin or equivalent DMSO and whole cell extracts obtained and subjected to native gel electrophoresis alongside heat shocked and medium only controls. 50 μ g of extract was run on a 3 to 20% gradient non-denaturing gel, and blotted for HSF-1. Monomeric and trimeric forms of HSF-1 are indicated, as is the Hsp90/HSF-1 complex present in resting cells. **Nil**, medium only; **HS**, heat shock; **C**, curcumin 10 μ M for 2 hours; **V**, Equivalent vehicle treatment (DMSO) for 2 hours.

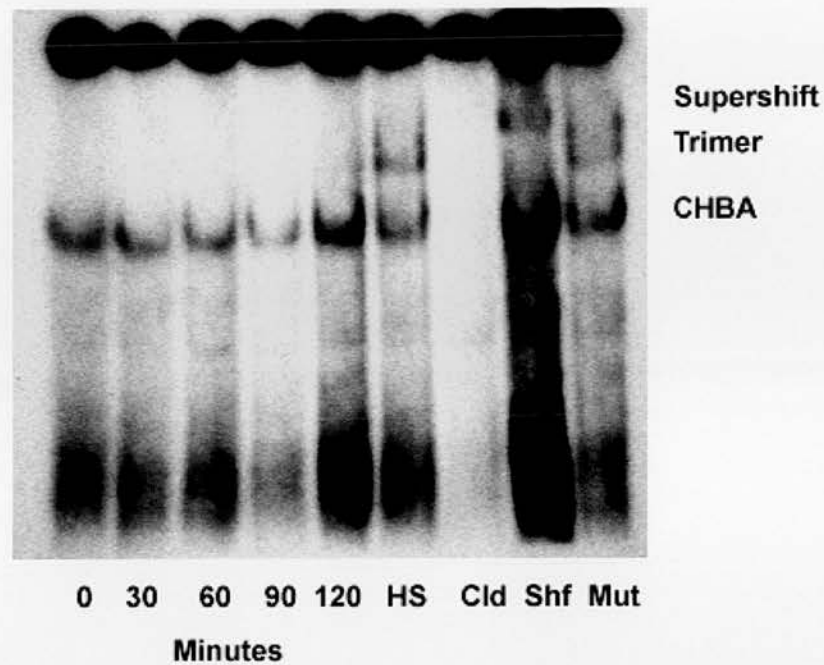


Figure 4.3 Non-toxic curcumin treatment does not induce trimeric HSF-1 DNA binding.

Cells were treated with 10 μ M curcumin for increasing amounts of time (0-120 minutes as indicated) and nuclear cell extracts were obtained. Gel mobility shift assay was used to assess HSE binding activity. For comparison a heat shocked cell lysate was run to demonstrate the active trimeric form of HSF-1 (**HS**). Cold native probe (**Cld**), antibody supershift (**Shf**) and cold mutant probe competition (**Mut**) controls are indicated and were performed using a heat shocked cell lysate. **CHBA**, constitutive HSE binding activity; **Trimer**, transcriptionally active trimeric form of HSF-1; **Supershift**, supershifted trimer position.

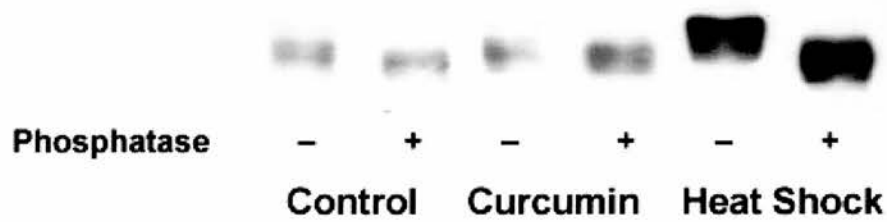


Figure 4.4 Non toxic curcumin treatment does not cause hyperphosphorylation of HSF-1.

Whole cell lysates were collected from cells treated with curcumin 10 μ M for 2 hours and from control and heat shocked cells. 20 μ g aliquots of each sample were treated either with lambda phosphatase or with buffer (+/- respectively) for 15 minutes and then run in parallel on a 7.5% gel and probed for HSF-1.

4.2.3 Effect of HSF-1 inhibition on HO-1 induction by Curcumin

To demonstrate that HO-1 induction by curcumin does not depend on HSF-1, experiments were performed using quercetin, an HSF-1 inhibitor. Quercetin was found to inhibit the induction of Hsp70 by heat shock when administered at 50 μ M. In contrast, the induction of HO-1 by curcumin was not reduced by quercetin treatment. Indeed, quercetin itself induced HO-1 and the combination of quercetin and curcumin had an additive effect on HO-1 induction (figure 4.5).

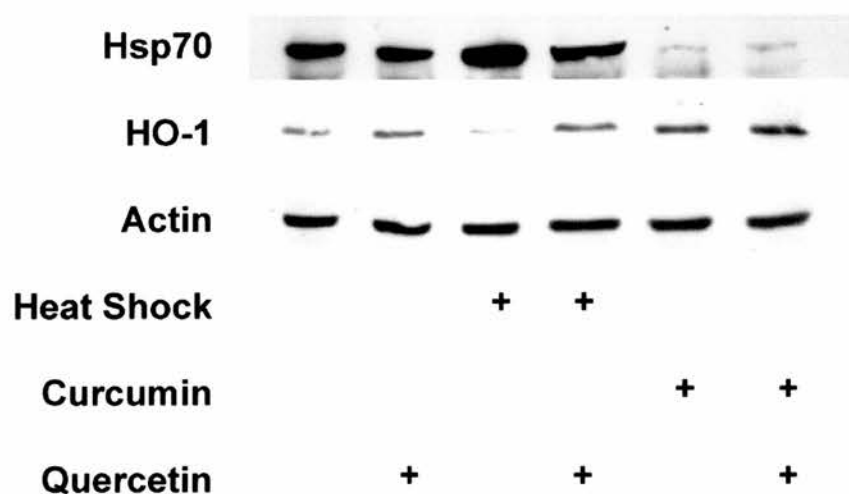


Figure 4.5 Quercetin does not inhibit the induction of HO-1 by 10 μ M curcumin.

HUH7 cells were treated with 50 μ M quercetin for 1 hour prior to and during curcumin exposure (curcumin 10 μ M or equivalent DMSO for two hours) or heat shock (43°C for 45 mins). Subsequently the medium was changed and the cells were recovered for 6 hours. Whole cell lysates were collected and then run on a 10% gel and probed for HO-1 and Hsp70.

4.3 Discussion

Initial experiments have shown that curcumin induced HO-1 and increased Hsp70 mRNA levels, both of which could potentially result from HSF-1 activation. For this reason, the effect of curcumin on HSF-1 in hepatocytes was examined. Curcumin was found to induce nuclear localisation of HSF-1 only after treatment with toxic doses. This was consistent with published results in the literature regarding HSF-1 and curcumin. Dunsmore found increased nuclear localisation of HSF-1 in HeLa cells after treatment with 30 μ M curcumin (Dunsmore 2001), a dose which was associated with significant toxicity.

In view of the finding that curcumin treatment at 10 μ M increased Hsp70 mRNA levels (although not Hsp70 protein), the effects of curcumin on HSF-1 were investigated in detail at this dose. This dose did not produce a detectable change in nuclear localisation. However, it was desirable to confirm whether there were any other changes in HSF-1 which may have resulted in transcriptionally active HSF-1. Activation and acquisition of transcriptional activity by HSF-1 is a complex multi-step process and some compounds such as salicylate can cause partial activation (Sarge 1993, Jurivich 1995). For this reason, all of the activation steps were assessed. However, in all methods used there was no evidence of changes in HSF-1, such as trimerisation or hyperphosphorylation, that would permit transcriptional activation.

Thus this does not explain the increase in Hsp70 mRNA levels. The increase could result from the activation of other transcription factors, or it could be the result of some kind of stabilisation event, as discussed in chapter 3.

The final experiments performed on HSF-1 examined whether curcumin pretreatment had a priming effect on HSF-1, allowing it to be more readily activated by other stimuli. This type of effect had been noted by other groups examining other cells types, where HSF-1 activation by sodium arsenite was prolonged by curcumin treatment (Kato 1998). No such priming effect was found in human hepatocytes. The method used to assess this was western blotting for HSF-1, and examination of its electrophoretic mobility. This is not a very sensitive method, and it may have been difficult to identify minor differences between experimental groups.

The human HO-1 promoter contains an HSF-1 consensus binding sequence (Heat Shock Element – HSE). In the rat this is active and HO-1 is a heat responsive protein. However, in human the HSE appears to be at least silent and it may be inhibitory (Okinaga 1996, Chou 2005). Quercetin was used to inhibit HSF-1, to establish if the HO-1 induction by curcumin was HSF-1 dependent. Quercetin is a weak partial agonist of HSF-1 and does have effects on pathways other than HSF-1 (Davies 2000), but nevertheless it is a commonly used HSF-1 inhibitor. It was found to have no inhibitory effect on HO-1 induction by curcumin, suggesting that this is an HSF-1 independent effect. Furthermore, quercetin was found to increase HO-1 levels and to have an additive effect on HO-1 induction by curcumin. This would be consistent with the observation that HSF-1 can have an inhibitory effect on HO-1

expression in human cells (Chou 2005), such that inhibition of HSF-1 should increase HO-1 levels. Accordingly, heat shock was noted to reduce HO-1 protein levels in this study.

4.4 Conclusion

Curcumin does activate HSF-1 after treatment with high doses and this is associated with significant toxicity. However, there was no increase in the transactivating potential of HSF-1 after treatment with 10 μ M curcumin. Thus the effect on HO-1 protein and Hsp70 mRNA is not the result of increased HSF-1 activity. As discussed earlier (see chapter 3), increases in Hsp70 mRNA levels could be the result of mRNA stabilisation. The mechanism by which HO-1 is induced remained to be elucidated.

Chapter 5

Mechanism of HO-1 Induction by Curcumin

5.1 Introduction

Initial experiments with curcumin had demonstrated that it is a potent inducer of heme oxygenase-1 in human hepatocytes. This chapter explores the mechanisms through which this induction may occur.

Heme oxygenase-1 regulation is a complex and tightly regulated phenomenon. HO-1 is a stress responsive enzyme and it is upregulated in response to a wide variety of stressors including hypoxia (Lee 1997, Kacini 2000), heavy metals and oxidative stress (Alam 1989). However, as explored in the introduction, there is marked variation in the regulation of HO-1 between species and between cell types (Sikorski 2004).

Curcumin has previously been shown to have certain properties which may underpin its ability to induce HO-1. It has been associated with generation of reactive oxygen species (Bhaumik 1999), which are toxic, and can induce HO-1, although the link has not been previously examined. Curcumin has also been shown to activate Nrf2 and the antioxidant response element (Balogun 2003), which will also induce HO-1. MAP kinases also have a role in the regulation of HO-1 induction (Kietzmann 2003), and curcumin has varied effects on MAP kinases depending on cell line and treatment conditions (Jobin 1999, Balogun 2003, Squires 2003).

The diverse effects attributed to curcumin, as described in the general introduction, and the variable responses seen in different cell lines suggest that curcumin may act

through an indirect pathway to induce HO-1, rather than a specific receptor mediated mechanism. Indeed, as HO-1 responds to many varied stimuli, the potential trigger mechanisms through which curcumin may act to induce HO-1 are similarly varied. However, the wealth of information on HO-1 induction by other agents and the effects of curcumin detailed above allowed identification of key mechanisms that were implicated in the mechanism of HO-1 induction by curcumin in human hepatocytes. For this reason, work to understand this mechanism concentrated on reactive oxygen species and downstream signals triggered by ROS generation – namely MAP kinases and the transcription factors Nrf2 and HIF-1 α .

Aim: To establish the cellular mechanisms through which curcumin induces HO-1 in human hepatocytes

5.2 Results

5.2.1 Curcumin induces ROS production

Cells were treated with increasing doses of curcumin and ROS production was measured by two different methods. Using H_2DCFDA the ROS generation was measured continuously for two hours. ROS were generated at a high rate within a minute of commencing curcumin treatment, and continued to be produced for the entire period studied, although the rate slowed as time progressed. In HUH7 cells, ROS production was maximal after treatment with 10 to $25\mu M$ curcumin, but near basal levels after treatment with $50\mu M$ (figure 5.1A). Addition of the protonophoric uncoupler CCCP to cells treated with $10\mu M$ curcumin prevented ROS production, suggesting it had a mitochondrial origin. Vehicle (DMSO) had no effect on ROS generation. The experiment was repeated on three occasions, with 6 replicates on each occasion. It was performed twice in isolated human hepatocytes, where similar results were obtained, except that after treatment with $50\mu M$ of curcumin, ROS generation was also elevated, and indeed was greater than that seen with 10 and $25\mu M$ (figure 5.1B).

The above experiment was repeated in HUH7 cells using the ROS sensitive dye dihydroethidium (figure 5.2). There was a significant induction of ROS in a dose dependent manner. This was consistent with the previous results after treatment with 10 and $25\mu M$ curcumin, but the greatest level of ROS generation was seen after treatment with $50\mu M$ curcumin.

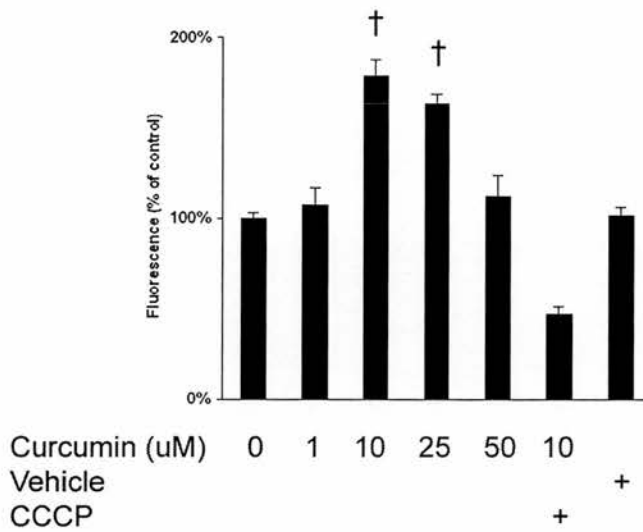
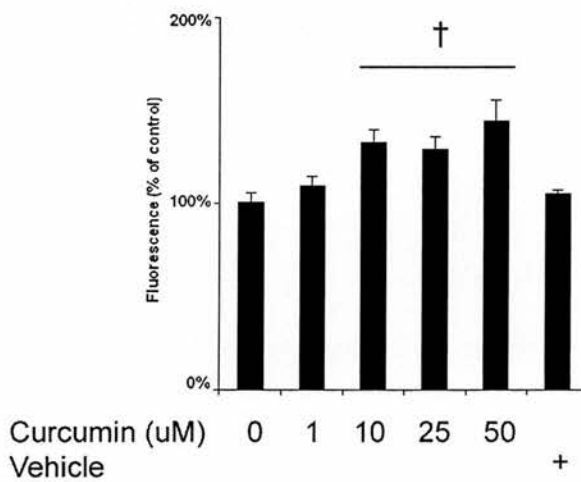
A**B**

Figure 5.1 Curcumin induces ROS production in human hepatocytes.

A. HUH7 cells. **B.** Isolated primary hepatocytes. ROS production was measured continuously using H_2DCFDA . Data shown represents total ROS production after one hour of treatment, as means \pm SEM of 6 repeats. Each experiment was performed 3 times with similar results. **CCCP** 10 μ M CCCP; **Vehicle** DMSO equivalent to 10 μ M curcumin. † = $p < 0.05$ vs nil control and vehicle.

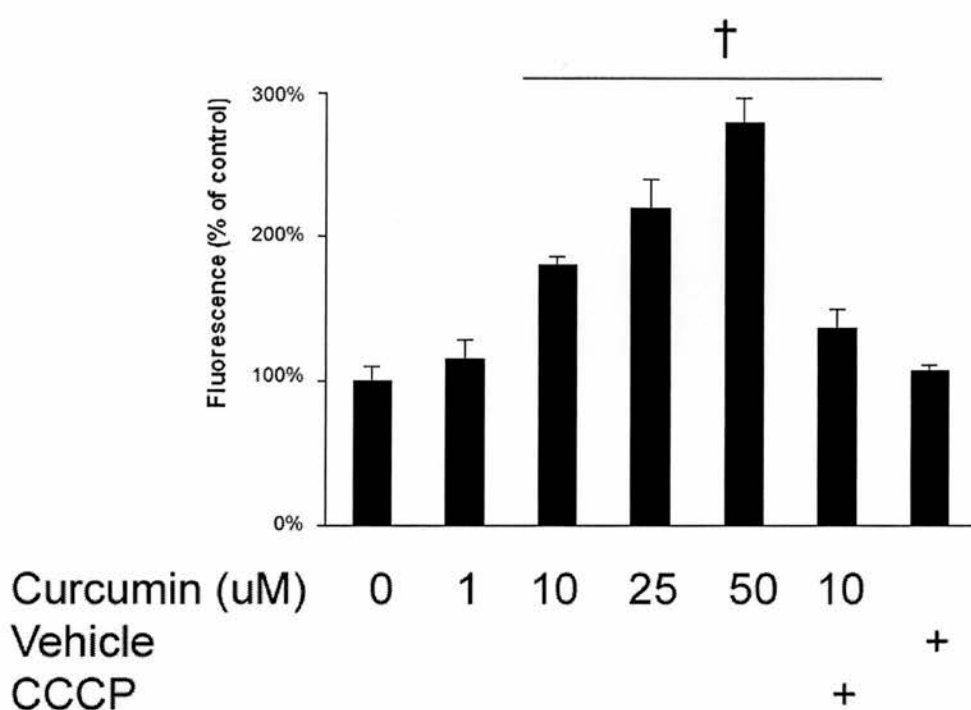


Figure 5.2 ROS production in HUH7 cells following curcumin treatment.

HUH7 cells were treated with curcumin and total ROS production at 4 hours measured with dihydroethidium. Curcumin induced ROS production in doses from 10-50 μ M. Each bar represents the mean \pm SEM of six repeats from one experiment. Each experiment was performed 3 times with similar results. **CCCP** 10 μ M CCCP; **Vehicle** DMSO equivalent to 10 μ M curcumin. † = $p < 0.05$ vs nil and vehicle control.

5.2.2 Curcumin treatment results in partial mitochondrial depolarisation

In view of a possible mitochondrial origin for the ROS production, curcumin induced changes in the mitochondrial membrane potential were measured. This was performed using two different methods – MitoTracker green and red dye, and DiOC6. Curcumin treatment with 10-50 μ M curcumin led to loss of the mitochondrial membrane potential as measured by MitoTracker red and green dye ($p < 0.01$, figure 5.3A). This was confirmed with use of DiOC6, which similarly demonstrated depolarisation of the mitochondria at 25-50 μ M, although using this method, 10 μ M had no effect, and the degree of depolarisation was much less pronounced (figure 5.3B).

5.2.3 Induction of HO-1 by Curcumin is mediated through Nrf2

Nrf2 is an oxidant-responsive transcription factor that has previously been shown to be involved in curcumin mediated induction of HO-1. In light of this and the above findings on ROS generation, Nrf2 was measured following curcumin treatment. Total cellular Nrf2 was increased following curcumin treatment, in doses of 10-25 μ M. This was the result of *de novo* synthesis, and it could be prevented by inhibiting transcription or translation with actinomycin D and cyclohexamide respectively (figure 5.4A).

The induction of Nrf2 by 10 μ M curcumin was assessed in the presence of CCCP, to examine if ROS generation had a role in increasing Nrf2. The increase in Nrf2 with

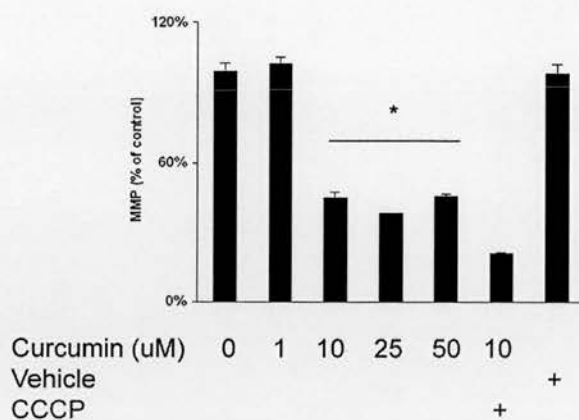
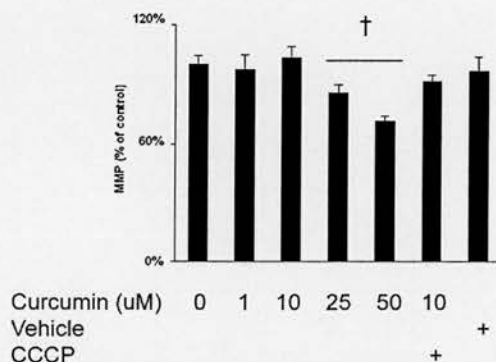
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Figure 5.3 Curcumin induces loss of the mitochondrial membrane potential.

A. Mitotracker red and green were used to measure the mitochondrial membrane potential (MMP). HUH7 cells were treated with curcumin for one hour. 10-50 μ M curcumin reduced the MMP (* = $p < 0.01$ vs nil control). **B.** DiOC6 was used to measure the MMP in HUH7 cells treated as above. 25-50 μ M curcumin lead to reduced MMP († = $p < 0.05$ vs nil control). Data represents the mean \pm SEM of six independent experiments. **CCCP** 10 μ M CCCP; **Vehicle** DMSO equivalent to 10 μ M curcumin.

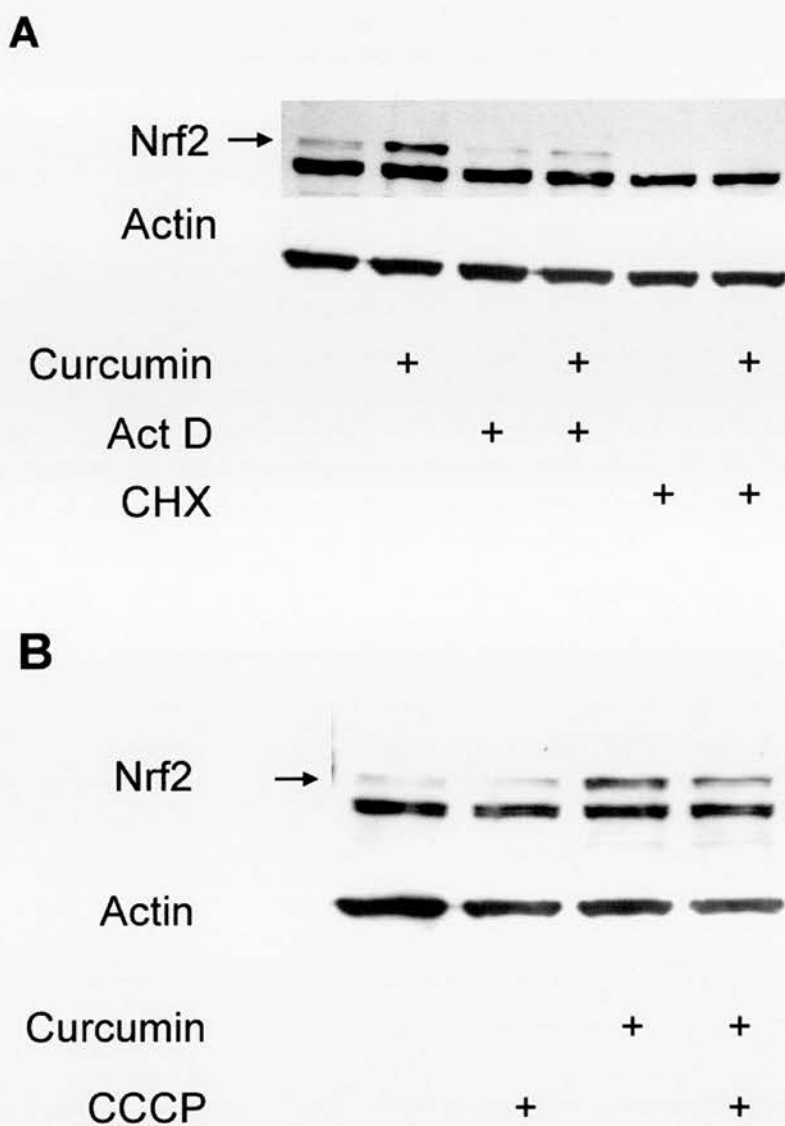


Figure 5.4 Curcumin induces HO-1 through Nrf2.

A. Nrf2 (upper band, arrowed) was measured in whole cell lysates from cells treated with 10 μ M curcumin for 3 hours. Nrf2 was strongly induced by curcumin treatment, and this was inhibited by treatment with actinomycin D (5 μ g/ml) or cyclohexamide (10 μ g/ml). **B.** Nrf2 (upper band, arrowed) induction by 10 μ M curcumin was partially inhibited by CCCP (10 μ M), which prevents ROS production.

curcumin treatment was more muted with CCCP treatment (figure 5.4B), suggesting that ROS may be involved in the upregulation of Nrf2.

The contribution of Nrf2 towards mediating the HO-1 induction seen with curcumin treatment was assessed using siRNA. The use of siRNA led to a reduction in Nrf2 levels following curcumin treatment, and this was found to partially abrogate the induction of HO-1 by curcumin. Using this method, control siRNA and siRNA for the transcription factor (HIF-1 α) had no effect, whilst Nrf2 siRNA did partially inhibit the induction of HO-1 by curcumin (figure 5.5).

5.2.4 Hypoxia Inducible Factor 1 α does not contribute to HO-1 activation

Activation of HIF-1 α following curcumin treatment was assessed by use of a HIF-1 α sensitive reporter containing three repeats of the HIF response element. Transfected HUH7 cells were treated with 10 μ M curcumin or vehicle for 6 hours and reporter activity measured. Cobalt chloride was used as a positive control. Curcumin treatment led to a reduction in reporter activity to 62% of control cells, which was similar to the effect of the vehicle (58% of controls, figure 5.6). The contribution of HIF-1 α was also assessed by siRNA (see figure 5.5) and by EMSA, but in keeping with the reporter results, there was no effect.

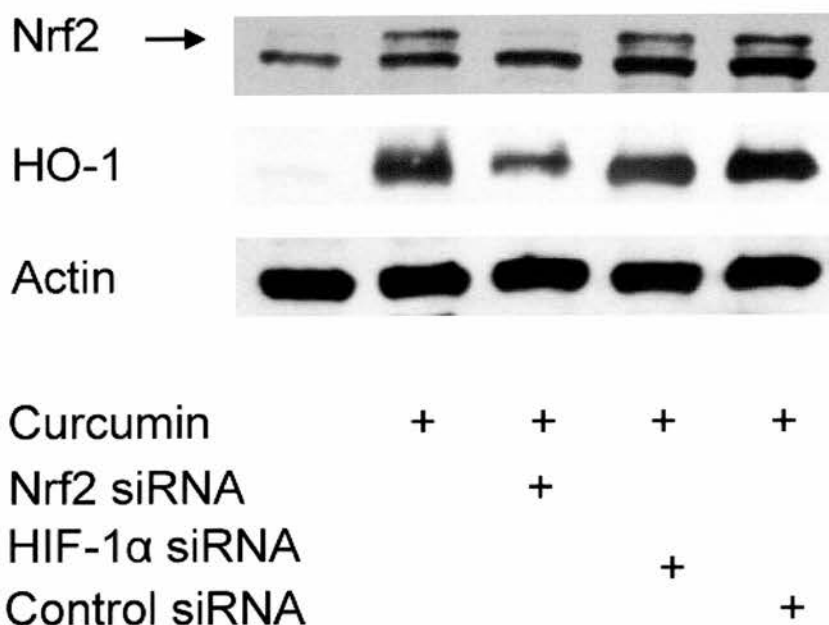


Figure 5.5 siRNA for Nrf2 but not HIF-1α reduces the HO-1 induction by curcumin.

HUH7 cells were treated with siRNA for either Nrf2, HIF-1α or a control sequence, and 48 hours later treated with curcumin (10μM) for 6 hours. Whole cell lysates were collected and a western blot for HO-1 and Nrf2 (upper band, arrowed) was performed. Blot shown is representative of 3 independent repeats.

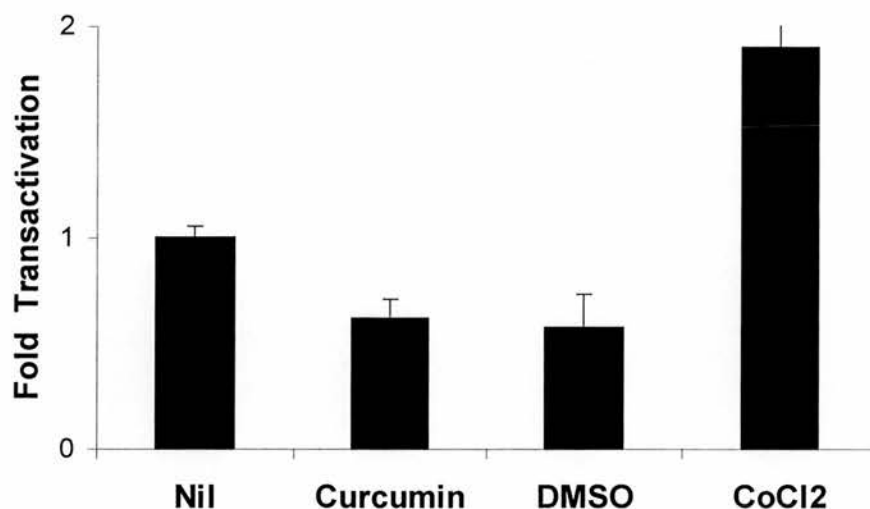


Figure 5.6 HIF-1 α activity is not increased by curcumin treatment.

HUH7 cells were double transfected with the HIF-1 α reporter and a pSV- β -Gal control construct and 48 hours later were treated with curcumin (10 μ M) for 5 hours and then lysed. Results are corrected for transfection efficiency and expressed as fold transactivation compared with controls. Cobalt chloride (**CoCl₂**) was used as a chemical HIF-1 α stimulus. There was no significant difference between curcumin and the effect of vehicle alone. Data shown represent the mean \pm SEM of 5 independent repeats.

5.2.5 Antioxidants inhibit HO-1 induction by curcumin

Earlier experiments had demonstrated that curcumin led to production of ROS. To confirm the role of oxidative stress in the induction of HO-1 by curcumin, antioxidants were used. HUH7 cells were pretreated with the antioxidants N-acetylcysteine or vitamin E for 30 minutes and then continuously during treatment with 10 μ M curcumin for 6 hours. Both antioxidants led to a reduction in HO-1 levels following curcumin treatment (figure 5.7). Treatment with catalase also reduced the induction of HO-1 in a dose dependent manner, suggesting hydrogen peroxide as an important contributing ROS (figure 5.8).

5.2.6 PKC and p38 contribute to the induction of HO-1 by curcumin

Kinase inhibitors were used to examine the contribution of MAP kinase activity to the induction of HO-1 by curcumin. This included inhibitors of protein kinase C (PKC), p38 MAPK, PI3K and ERK. The treatment concentrations were taken from published studies, using the most specific inhibitors identified in the literature (Davies 2000). HO-1 induction was measured by HO-1 reporter activity following treatment with 10 μ M curcumin in the presence of the various inhibitors. Inhibition of PKC and p38 significantly reduced HO-1 induction (280% down to 168% and 184%, $p < 0.01$ and $p < 0.05$ respectively, figure 5.9). Inhibition of PI3 kinase also reduced the HO-1 induction, although this did not reach significance (280% to 231%, $p = 0.181$), and inhibition of ERK had no effect. The results obtained from using the reporter were confirmed by western blotting which confirmed dose dependent inhibition of

HO-1 induction with PKC inhibition (figure 5.10A). The p38 inhibitor (SB203580) was so potent that it was effective even at 2 μ M (figure 5.10B). Activation of p38 by curcumin was confirmed by western blotting for the phosphorylated active form (figure 5.11).

5.2.7 Curcumin inhibits phosphatase activity

Phosphatase activity was examined after curcumin administration (10 μ M). PP2A activity was significantly inhibited within 5 minutes of curcumin treatment and remained reduced for one hour (80% of basal activity, $p < 0.05$, figure 5.12). PP2C activity was unchanged by curcumin treatment. Tyrosine phosphatase activity was significantly reduced only after one hour of curcumin treatment (77% of basal activity, $p < 0.05$).

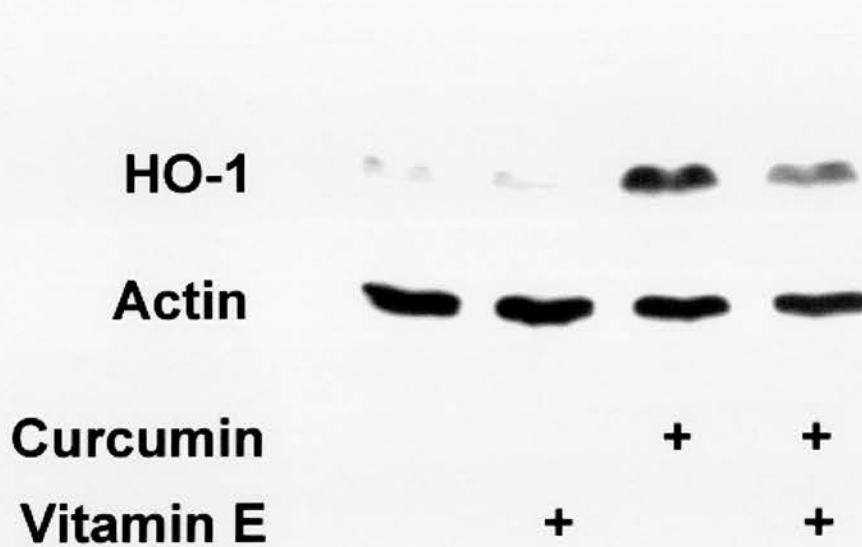
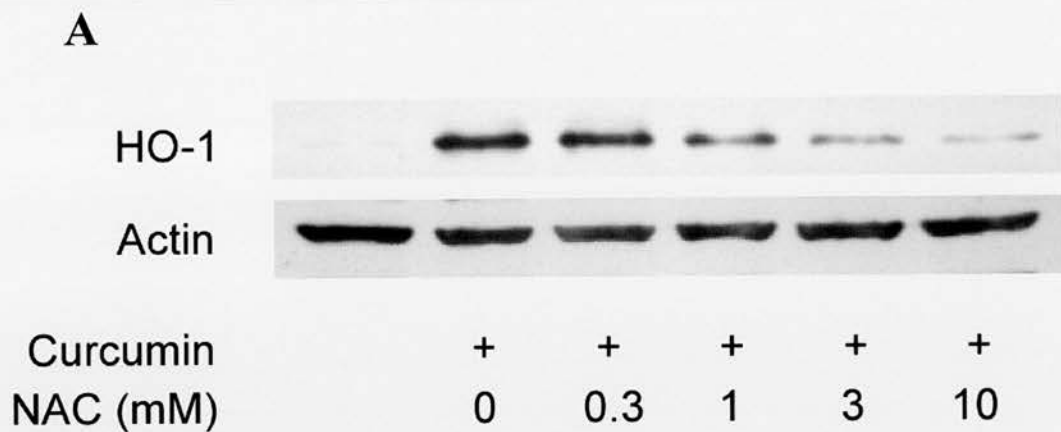


Figure 5.7 Antioxidants inhibit HO-1 induction by curcumin.

A. HUH7 cells were pretreated with increasing doses of N-acetylcysteine (NAC) for 30 minutes prior to treatment with 10 μ M curcumin. **B.** Pretreatment with 50 μ M vitamin E for 30 minutes reduced the HO-1 induction seen with 10 μ M curcumin. Blots shown are representative of three individual experiments.

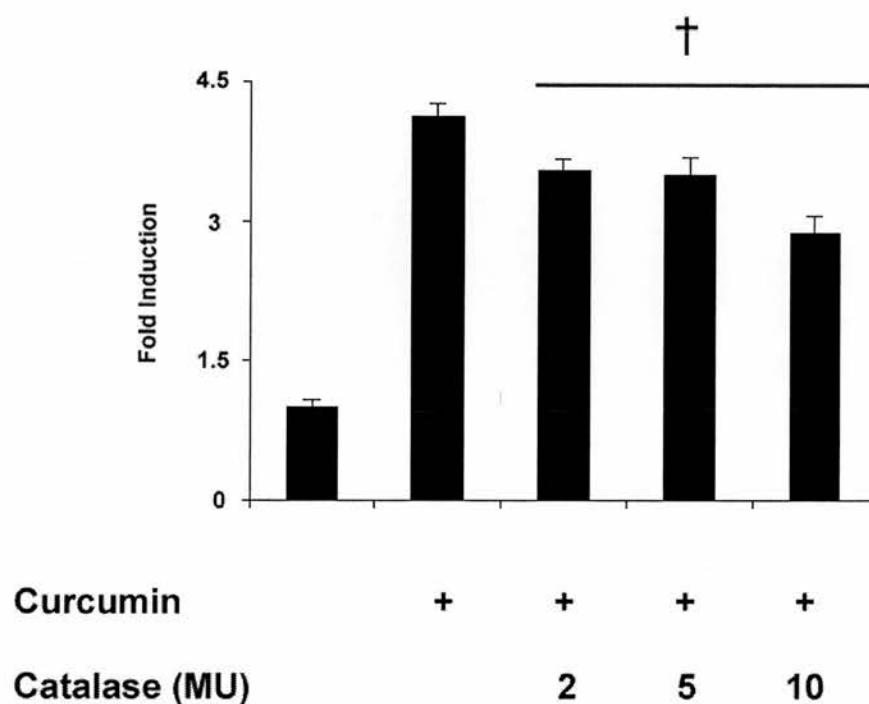


Figure 5.8 Catalase inhibits the induction of HO-1 by curcumin.

HUH7 cells were co-transfected with an HO-1 luciferase reporter (pHOGL3/11.6) and a control vector (PSV β Gal), and treated with 10 μ M curcumin in the presence of varying concentrations of catalase (2-10 thousand units, **MU**). Catalase treatment reduced the HO-1 reporter activity seen with curcumin treatment alone. † = $p < 0.05$ vs curcumin only.

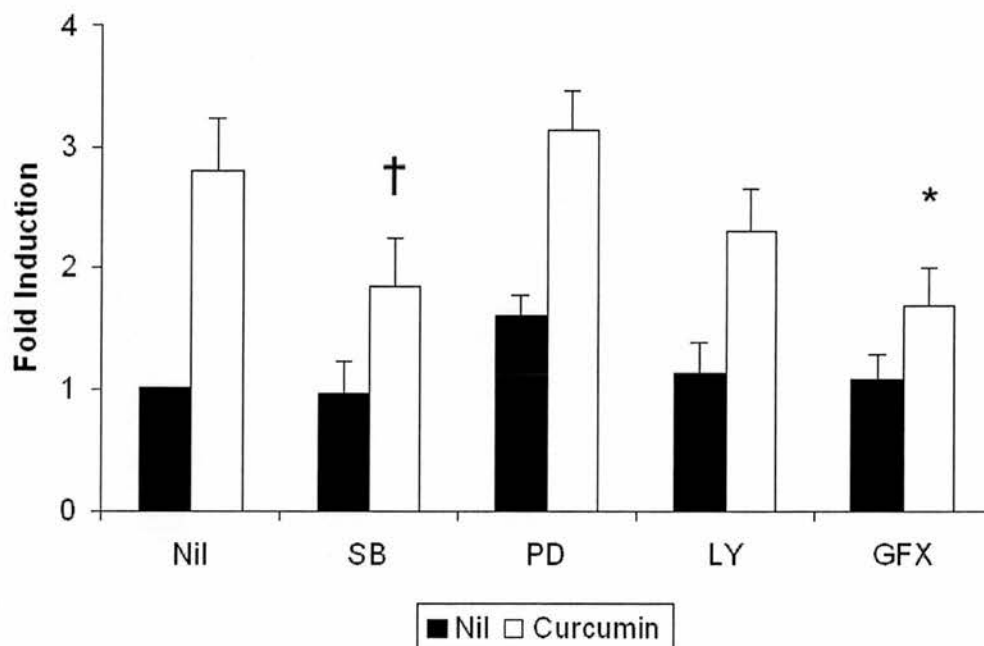
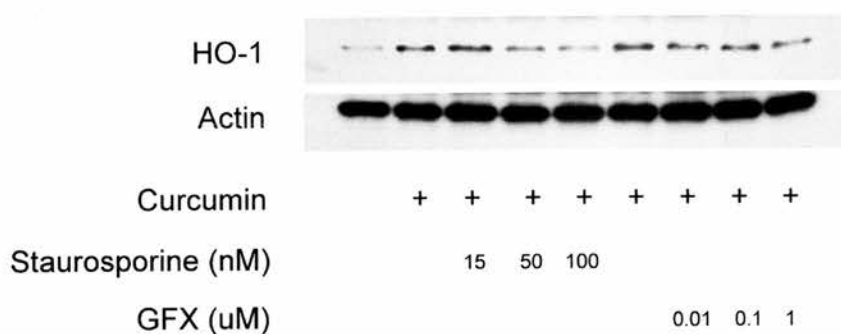


Figure 5.9 PKC and p38 contribute to HO-1 induction by curcumin.

HUH7 cells were transfected with pHOGL3/11.6 and the response to 10 μ M curcumin was measured in the presence of a panel of kinase inhibitors. **Nil** no inhibitor; **SB** 20 μ M SB203580, a p38 inhibitor; **PD** 5 μ M PD98059, an ERK inhibitor; **LY** 2 μ M LY294002, a PI3K inhibitor; **GFX** 5 μ M GF109203X, a PKC inhibitor. SB and GFX inhibited HO-1 induction by curcumin (* = $p < 0.01$ and † = $p < 0.05$).

A



B

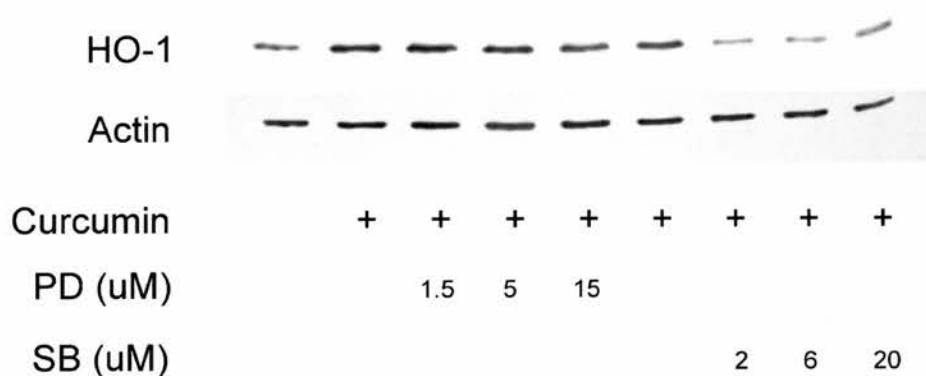


Figure 5.10 PKC and p38 are involved in the induction of HO-1 by curcumin.

A. Dose response of two different PKC inhibitors, staurosporine and GF109203X (**GFX**) showing inhibition of HO-1 the HO-1 inducing activity of 10μM curcumin.

B. Dose response of an ERK inhibitor (**PD** PD98059) and a p38 inhibitor (**SB** SB203580) showing the effect of p38 inhibition on HO-1 induction by 10μM curcumin.

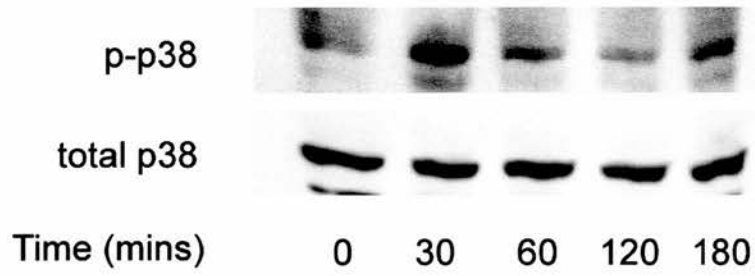


Figure 5.11 p38 MAPK is activated following curcumin treatment.

Western blot showing activation of p38 following treatment with 10 μM curcumin. p38 was activated after 30 minutes of curcumin treatment. **p-p38** phosphorylated (active) p38 MAPK. Total p38 is shown as a loading control.

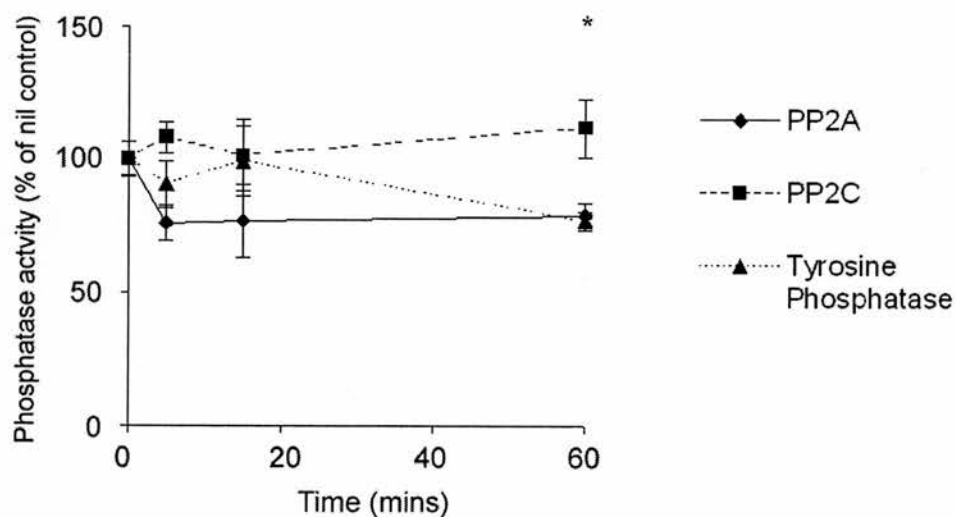


Figure 5.12 Curcumin inhibits protein phosphatase activity.

HUH7 cells were treated with 10 μ M curcumin and phosphatase activity was measured at 5, 15 and 60 minutes. **PP2A** and **PP2C** protein phosphatase 2A and 2C respectively. PP2A and tyrosine phosphatase activity were significantly reduced after one hour of treatment (*= $p < 0.05$). PP2C activity was unchanged. Data shown represent the mean \pm SEM of 8 independent repeats.

5.3 Discussion

Curcumin has many attributed cellular effects, and this chapter examines in detail the relevant mechanisms through which curcumin may act to induce HO-1.

Curcumin treatment induced ROS production in a dose responsive manner. These ROS may originate from the mitochondria, as they could be inhibited by the uncoupler CCCP, and curcumin treatment was also associated with mitochondrial depolarisation, which is often linked with high level mitochondrial ROS production. Curcumin has been found to generate superoxide in cell lines that are sensitive to its pro-apoptotic properties (Khar 2001) and, in isolated rat mitochondria, curcumin induces mitochondrial depolarisation in an ROS dependent manner (Ligeret 2004). This is consistent with the earlier results demonstrating caspase activation with increasing doses of curcumin (chapter 3), reinforcing that treatment with curcumin in high doses can lead to apoptosis through a classical, mitochondria-dependent pathway, likely as a result of its ROS inducing capabilities.

The two methods used for ROS measurement produced slightly different results. This is likely to reflect differences in sensitivity to the different ROS species. H₂DCFDA is more sensitive for hydrogen peroxide and hydroxyl radicals, whilst dihydroethidium is more sensitive for superoxide although neither is species specific (Lee 1998, Zhang 2001).

Slightly different results were also found when using isolated human hepatocytes and HUH7 cells. There was consistent induction of ROS after treatment with 10 and 25µM of curcumin, but divergent results when measuring ROS generation after treatment with 50µM. The reason for this is unclear. There are many differences between an immortalised cell line and primary cultures, and it may be that the fresh primary cells retain some unspecified enzymatic activity which is lost in the HUH7 cells. The results obtained using dihydroethidium in HUH7 cells are consistent with those obtained with H₂DCFDA in the primary hepatocytes, raising the possibility that there is a difference in the relative abundance of different ROS species being produced in the two different cell types. However, the level of endogenous antioxidants was not measured, which may vary between the cell types and contribute to the observed differences.

The ROS generating properties of curcumin reflect its chemical structure, and certain other flavonoids can also generate ROS (Pan 2005), although they are more often associated with antioxidant activity (Paola 2005, Yamaguchi 2005). Ligeret examined the effects of curcumin derivatives on rat liver mitochondria, and found that the phenolic group was essential to effect depolarisation of the mitochondria (Ligeret 2004). This allows curcumin to reduce Fe³⁺ to Fe²⁺, which subsequently allows generation of hydroxyl radicals by the Fenton reaction. Thus Ligeret proposes that it is the reductive “antioxidant” properties of curcumin which results in increased ROS production. If the Fenton reaction was a contributor to ROS generation then could be tested using an iron chelating agent, however, this was not directly tested in this work. If the Fenton reaction were involved in the induction of

HO-1 by curcumin, then such induction should have been prevented by catalase.

However, despite the high concentrations of catalase used in these experiments, there was only an attenuation of HO-1 induction. This suggests that hydrogen peroxide may contribute to the rise in HO-1, but it is not the sole responsible reactive oxygen species.

In examining the transcription factor Nrf2, it was found to be induced by curcumin treatment. Nrf2 is rapidly induced by oxidative stress, and binds to consensus sequences in the HO-1 promoter to mediate its induction. Nrf2 has previously been shown to be involved in the HO-1 induction by curcumin (Balogun 2003), although this was examined in non-human kidney cells. There are significant interspecies differences in the HO-1 promoter (Sikorski 2004), and this work confirms this finding for the first time in human cells, and for the first time using siRNA. Attempts were made to perform gel shift assays to detect Nrf2 DNA binding, however, despite several methods being tested, this was unsuccessful. This would have been helpful as another layer of evidence to demonstrate Nrf2 activation by curcumin, although this has been shown previously (Balogun 2003). The presence of increased levels of Nrf2 after curcumin treatment and the reduction in HO-1 levels with the Nrf2 siRNA supports the activation of Nrf2 as a mechanism through which curcumin induces HO-1, even in the absence of gel shift data.

Hypoxia inducible factor 1 α was examined as a potential contributor to the induction of HO-1. It is as its name suggests a hypoxia sensitive transcription factor, and it is a potent stimulator of HO-1 production (Lee 1997). It has also been shown to respond

to chemical oxidative stress in the liver (Tacchini 2002). It was for this reason that HIF-1 α activity was studied. However, whilst the human HO-1 promoter contains a consensus binding sequence for HIF-1 α , it has recently been established that hypoxia does not induce HO-1 in human tissues, although it does in other species (Kitamuro 2003). Whilst this cannot be attested to from the experiments performed here, HIF-1 α does not appear to be activated by curcumin, as evidenced by the lack of reporter activity. This finding was corroborated by the lack of effect of HIF-1 α siRNA on HO-1 induction by curcumin.

These experiments demonstrate for the first time that HO-1 induction by curcumin can be inhibited by the use of the antioxidants N-acetylcysteine and vitamin E. It is proposed that curcumin generates mitochondrial ROS and that this is the trigger for Nrf2 induction and subsequent upregulation of HO-1. If curcumin is administered in toxic doses the mitochondria undergo the permeability transition pore, and the cell is destined to apoptose. However, in non-toxic doses curcumin generates sufficient ROS to trigger activation of Nrf2, PKC and p38, and inhibit phosphatase activity, all of which contribute to HO-1 induction (figure 5.13). This is consistent with experiments performed using the garlic extract DAS, another HO-1 inducer which has recently been found to stimulate ROS production (Gong 2004).

Scapagnini found, in astrocytes, that curcumin was associated with toxicity at doses of 50 μ M and over, and that this could be inhibited by the addition of N-acetylcysteine, which is consistent with our findings regarding curcumin-induced ROS generation and its apparent association with cellular toxicity. However, when

examining the effect of N-acetylcysteine on HO-1 induction by curcumin, Scapagnini found no apparent inhibition when used at a concentration of 1mM (Scapagnini 2002). This may a result of the dose of N-acetylcysteine used. The results from this chapter show that curcumin is a very potent inducer of ROS. Furthermore, there was a dose responsive decrease in HO-1 induction with increasing doses of N-acetylcysteine, which was only apparent in doses greater than

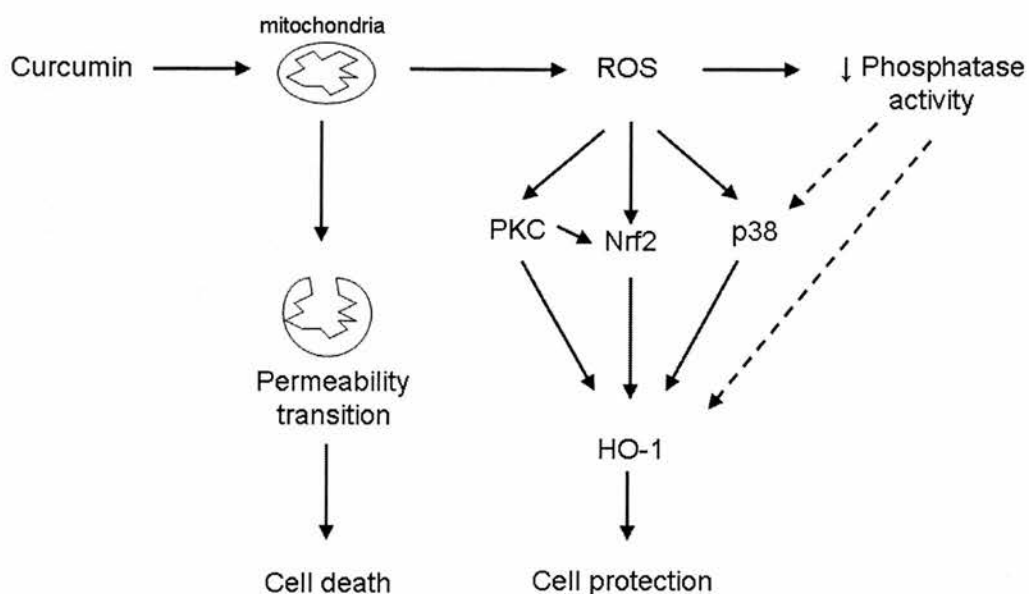


Figure 5.13 Proposed mechanisms through which curcumin induces HO-1.

Curcumin penetrates the cell and leads to ROS generation from mitochondria. If it is given in high doses ($>25\mu\text{M}$) the mitochondria undergoes the permeability transition pore and cell death ensues. If given at non toxic doses ($25\mu\text{M}$ and less), the ROS generated triggers Nrf2 accumulation and activation, activation of PKC and p38, and inhibition of protein phosphatases, all of which contribute to induction of HO-1 and protection of the cell from injury.

1mM. It is possible that in astrocytes, as in hepatocytes, the volume of ROS produced is too great to be quenched by 1mM N-acetylcysteine, and that higher doses are required. It remains possible that the high doses of N-acetylcysteine that were effective in hepatocytes (3 to 10mM) are exerting their effect through other changes in the cell rather than simply the antioxidant effect of the drug. However, vitamin E and catalase were able to reproduce the effect, supporting the contention that the induction of HO-1 by curcumin is a direct result of its ability to induce ROS production.

Glutathione is one of the cell's natural anti-oxidants and maintenance of reduced glutathione is important in the ability of a cell to resist oxidant-induced apoptosis (Boggs 1998, Si 1998). It has been shown that some anti-apoptotic proteins exert their protective effect through maintenance of glutathione, such as Bcl-2 (Voehringer 1999, Schor 2000). Curcumin has been shown to elevate glutathione levels in Jurkat cells and thymocytes (Piwocka 2001, Jaruga 1998). It is conceivable that elevation of glutathione could contribute to the cytoprotective properties of curcumin, in addition to increasing the level of HO-1. However, it is important to note that this elevation of glutathione was only seen after treatment with 50 μ M curcumin, and that this dose led to significant cell death in Jurkat cells (Piwocka 2001), in keeping with that seen in this work (chapter 3).

In addition to the effects of Nrf2, PKC and p38 activity are important in the induction of HO-1 by curcumin. Protein kinase C (PKC) is involved in activation of Nrf2, by phosphorylating it to allow dissociation from the cytoplasmic inhibitor

Keap1. This allows nuclear migration of Nrf2 where it binds to the requisite promoters (Bloom 2003). Whilst this would explain the contribution of PKC activity to HO-1 induction by curcumin, a direct effect on other transcription factors cannot be excluded.

p38 has previously been found to contribute to HO-1 induction by cadmium and curcumin, although the mechanism is unclear and it may be intimately linked with Nrf2 activation (Alam 2000, Balogun 2003). Balogun examined the contribution of MAP kinases to the induction of HO-1 by curcumin in porcine renal cells (Balogun 2003). p38 MAPK was found to be activated by curcumin treatment and inhibition of p38 was found to reduce the level of induction, consistent with the results of this chapter. Inhibition of ERK and JNK had no effect in that study, and the contribution of PKC was not examined.

Protein kinases are dependent on phosphorylation of threonine and tyrosine residues to stimulate their activity, the result of which is that control of cellular processes is regulated through a delicate balance of protein kinase and protein phosphatase activity. After having demonstrated the involvement of kinase activation in curcumin's induction of HO-1, cellular phosphatase activity was examined. It was found that PP2A was rapidly inhibited by curcumin and that tyrosine phosphatase activity was inhibited after one hour of treatment. It is likely that this plays a role in the HO-1 induction seen with curcumin, as the PP2A inhibitor okadaic acid is a potent HO-1 inducer (Immenschuh 2000). PP2A can be inhibited by ROS (Kim 2003), and although not directly tested, this would be consistent with the rapid

decrease seen in its activity, as ROS were generated constantly at high levels from within a minute of treatment. PP2A acts directly on p38 resulting in its inactivation (Alvarado-Kristensson 2005). This may represent part of the link between curcumin treatment and p38 activation, as the inhibition of PP2A activity would directly result in increased p38 activity.

PP2A also acts directly on caspase 3, leading to its inactivation (Alvarado-Kristensson 2005). Curcumin treatment leads to a dose dependent increase in caspase 3/7 activity (chapter 3), and although a dose response was not performed in this study, it is possible that increasing curcumin concentrations could cause greater inhibition of PP2A and hence contribute to the pro-apoptotic events which occur following high dose treatment.

5.4 Conclusion

Curcumin treatment results in ROS generation, activation of Nrf2 and MAP kinases and the inhibition of phosphatase activity in hepatocytes, and when curcumin is not administered in toxic doses, these multiple pathways converge to induce HO-1 (figure 5.13). Thus curcumin can be used at low doses to pharmacologically induce HO-1, and its induction is through generation of non-lethal levels of reactive oxygen species. Whilst several of these mechanisms have individually been demonstrated by other investigators, this is the first study to look at them all in combination and confirm their contribution to the induction of HO-1 by curcumin.

That curcumin could robustly induce HO-1 in hepatocytes without causing toxicity had been demonstrated in chapter 3. However, it remained to be seen whether the HO-1 induction caused by curcumin treatment could be used to pharmacologically precondition human hepatocytes.

Chapter 6

Curcumin and Cytoprotection

6.1 Introduction

The stress response is the cell's natural response to injury and underpins its ability to protect itself from further insults. Having found that curcumin can induce HO-1 in human hepatocytes, the next step was to investigate the protective effect of HO-1 induction by curcumin and assess its preconditioning potential.

In liver surgery there are two particular scenarios where preconditioning may be of benefit: resectional liver surgery and liver transplantation. During resectional liver surgery the inflow of blood to the liver may be temporarily interrupted for technical reasons by application of the Pringle manoeuvre, whereby the hepatic pedicle containing the hepatic artery and portal vein is clamped (Dixon 2005). This results in a period of warm ischaemia followed by subsequent reperfusion injury when the hepatic inflow is restored (figure 1.1). During liver transplantation, the liver is perfused with cold preservation solution, removed from the body and placed on ice for a period of time prior to reimplantation and restoration of its circulation. This therefore results in a period of cold ischaemia, followed by a brief period of warm ischaemia (while the liver is in the body cavity and anastomoses are being performed to restore vessel continuity), and then a period of reperfusion (figure 1.1).

HO-1 induction has been shown to be protective against ischaemia-reperfusion injury and cold preservation injury (Akamatsu 2004, Wang 2005, Uchida 2003). If curcumin is to be a clinically useful preconditioning agent it must be able to protect

hepatocytes from both of these insults. This has not previously been tested in human hepatocytes using curcumin as an HO-1 inducing agent.

Aim: To establish whether HO-1 induction by curcumin can precondition human hepatocytes in models simulating ischaemia reperfusion injury and cold preservation injury.

6.2 Results

6.2.1 Development of *in vitro* models of ischaemia reperfusion injury

Initial experiments aimed to establish a reliable *in vitro* model of ischaemia-reperfusion injury. Several models were explored, including: sodium arsenite; anoxia and reperfusion; ultraviolet light; simulated chemical ischaemia reperfusion; glucose oxidase with or without prior anoxia; and hydrogen peroxide. A significant contributor to the damage of reperfusion injury is oxidative stress, and each model mimics this to some degree. Whilst examining the various models, hemin was used as a positive control, representing a known HO-1 inducer which is cytoprotective (Clark 2000, Attuwaybi 2004). Thus a model would be rejected on technical grounds if it was unable to demonstrate protection by hemin.

Sodium arsenite (SA) was the first model used, initially in combination with propidium iodide and annexin V flow cytometry as an outcome measure. However, this method was unstable, with SA treatment producing inconsistent levels of cell death, despite titrations of doses from 1 to 5mM. Two attempts were made to identify a preconditioning effect, but there was no significant difference between treatment with either hemin or curcumin (figure 6.1).

It was considered that the process of cell harvesting for flow cytometry and the prolonged preparation process may have been damaging to the cells. For this reason, a modified MTT assay was used as a measure of survival (see chapter 2). Initial

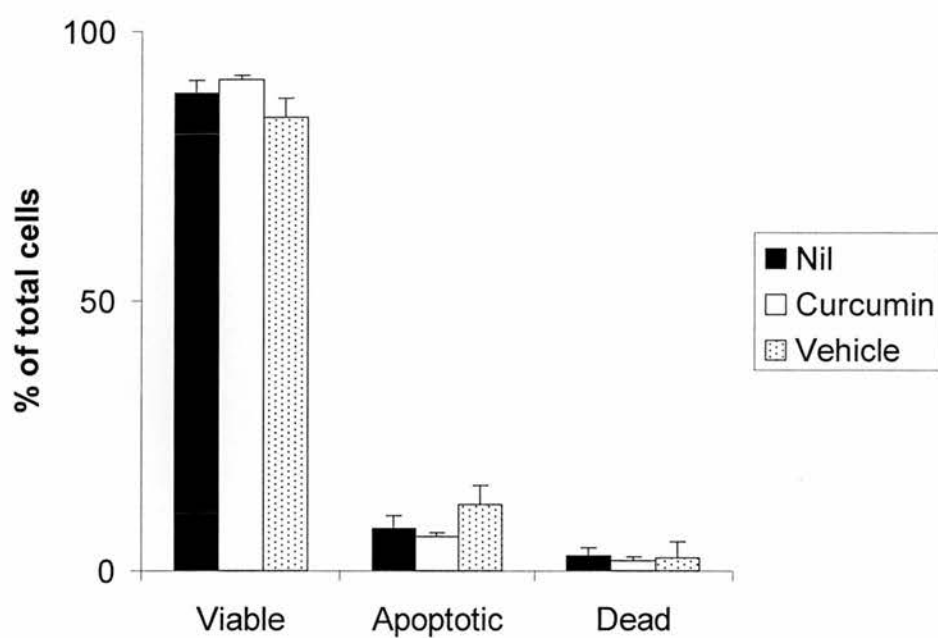


Figure 6.1 Curcumin does not protect from arsenite mediated injury.

HUH7 cells were treated with 5mM sodium arsenite for 30 minutes after pretreatment with either 10 μ M curcumin or corresponding vehicle. Cell viability was then assessed using propidium iodide and annexin V flow cytometry. There was no significant difference between the treatment groups.

testing found that 5mM SA reproducibly reduced survival by approximately 40%. Experiments performed subsequently using prior treatment with hemin or curcumin showed no preconditioning effect. This was performed twice with the same result (figure 6.2). Thus sodium arsenite was rejected as a model.

The next model tested was anoxia and reperfusion. Cells were placed in a sealed chamber at 37°C and subjected to anoxia by flooding the chamber with a 5% CO₂/95% N₂ mixture. After five hours, the cells were returned to normal culture conditions for four hours and survival was assessed by either MTT assay or propidium iodide flow cytometry. This model was examined using primary cultures of isolated human hepatocytes as well as HUH7 cells. Five hours of anoxia resulted in insufficient cell death to distinguish any differences between treatment groups (figure 6.2). The period of anoxia was extended to 16 hours and tested with HUH7 cells, but similar results were obtained and this model was abandoned.

Ultraviolet light is toxic through generation of intracellular reactive oxygen species which damage DNA (Zhang 1997). UVC was used, in doses of 50, 100, 150 or 200J. Survival was assessed by assaying released LDH in the culture medium 16 hours after UVC treatment. There was a clear dose dependent increase in cell death with increasing doses of UVC. 50J was chosen for further study, and 10µM curcumin was found to be protective against injury, reducing LDH release to 75% that of untreated cells. Furthermore, this protective effect was prevented by addition of the HO-1 inhibitor zinc protoporphyrin (ZnPPiX), suggesting that the protective effect was due to heme oxygenase activity ($p < 0.05$, figure 6.3). This experiment was performed in

triplicate on two occasions, and the results combined to allow statistical analysis. However, due to the prolonged time between injury and assessment of survival/death, there was significant potential for confounding due to various factors including cell proliferation, rendering the model flawed, and so it was not pursued further (see discussion).

Simulated ischaemia reperfusion was achieved by use of a buffer containing deoxyglucose and sodium dithionite, with survival after treatment assessed by MTT assay. This method was taken from published studies examining ischaemia reperfusion in cardiomyocytes (Cumming 1996). This showed promising results with a clear and consistent dose responsive decrease in survival with increasing duration of exposure to the buffer (figure 6.4). However, this method was piloted at the same time as the glucose oxidase/hydrogen peroxide models, and it was decided to focus mainly on the latter.

Models using glucose oxidase or hydrogen peroxide to generate reactive oxygen species were tested simultaneously. Glucose oxidase catalyses the degradation of glucose, generating hydrogen peroxide in the process. This generates a constant level of hydrogen peroxide over the treatment period. In contrast, bolus administration of hydrogen peroxide has a short half-life. It was found that both curcumin and hemin were capable of protecting HUH7 cells in both models. The glucose oxidase model proved to be more brittle than bolus administration of hydrogen peroxide, with greater variability in the degree of cellular injury between experiments. Thus although both models were used successfully, hydrogen peroxide was more

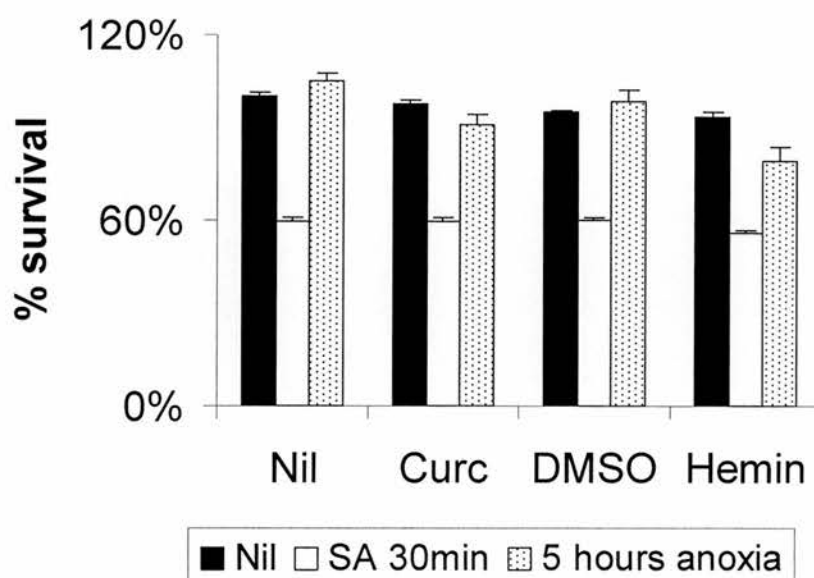


Figure 6.2 Arsenite toxicity and anoxia and reperfusion are inadequate models to test HO-1 mediated cytoprotection by curcumin.

Isolated human hepatocytes were treated with 10 μ M curcumin, vehicle (DMSO) or 20 μ M hemin for 8 hours. They were then subjected to either 5mM sodium arsenite for 30 minutes or anoxia for 5 hours. Survival was assessed by MTT assay. Hemin was unable to precondition hepatocytes in these models. Data represent the mean \pm SEM of 6 replicates.

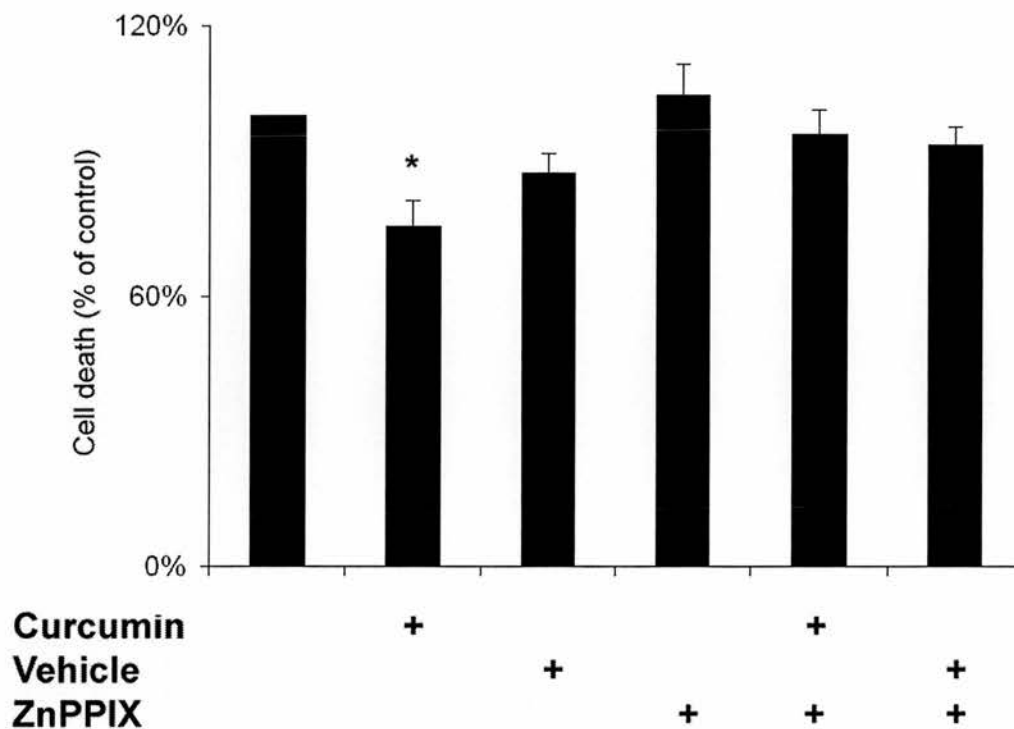


Figure 6.3 Pretreatment with 10μM curcumin protects against UVC mediated cell toxicity.

HUH7 cells were pretreated with 10μM curcumin for 8 hours prior to UVC irradiation (50J). They were then recovered for 16 hours in the presence or absence of the HO-1 inhibitor ZnPPiX (50μM). Curcumin pretreatment protected against cell death and this protection was removed by the presence of ZnPPiX. *p<0.05 vs untreated control, vehicle or curcumin and ZnPPiX.

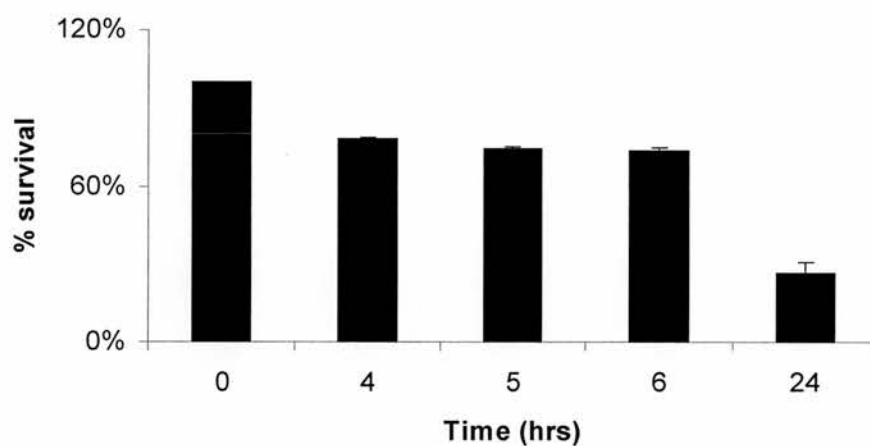


Figure 6.4 Simulated ischaemia reperfusion buffer tested in HUH7 cells.

A chemical buffer simulating ischaemia-reperfusion was piloted in HUH7 cells.

There was a clear relationship between exposure time and cell survival. Data shown represent mean \pm SEM of 6 replicates from a single experiment. The experiment was performed twice.

predictable. A variation on this model, subjecting the cells to a period of anoxia prior to glucose oxidase treatment was also tested. However, the addition of anoxia failed to induce any additional cellular injury in the HUH7 cells, and it was not implemented as part of the model.

6.2.2 Hemin protects in a model of oxidative injury

HUH7 cells were pretreated with 20 μ M hemin for 6 hours, which is sufficient to induce HO-1. The cells were then treated with 90mU/ml of glucose oxidase. Other groups included treatment with ZnPPiX (50 μ M, an HO inhibitor), bilirubin (1 μ M) or dichloromethane (0.1%, a chemical source of carbon monoxide). Hemin was found to significantly protect against injury ($p<0.01$). This protection was reduced in the presence of HO-1 inhibition ($p<0.05$), and could be restored by the addition of carbon monoxide, suggesting that the protection was a direct result of the HO-1 induction by hemin (figure 6.5).

6.2.3 Curcumin protects in a model of oxidative injury

Curcumin was evaluated for a preconditioning effect in HUH7 cells using bolus administration of 1mM hydrogen peroxide as a model of oxidative injury. Curcumin pretreatment significantly improved cell survival compared with either no pretreatment or vehicle (25 ± 6 vs. 0 ± 3 and 3 ± 4 , $p<0.01$ and $p<0.05$ respectively, figure 6.6). This represented protection of one third of the cells that would otherwise have died. Further experiments were performed to establish the contribution of HO-1

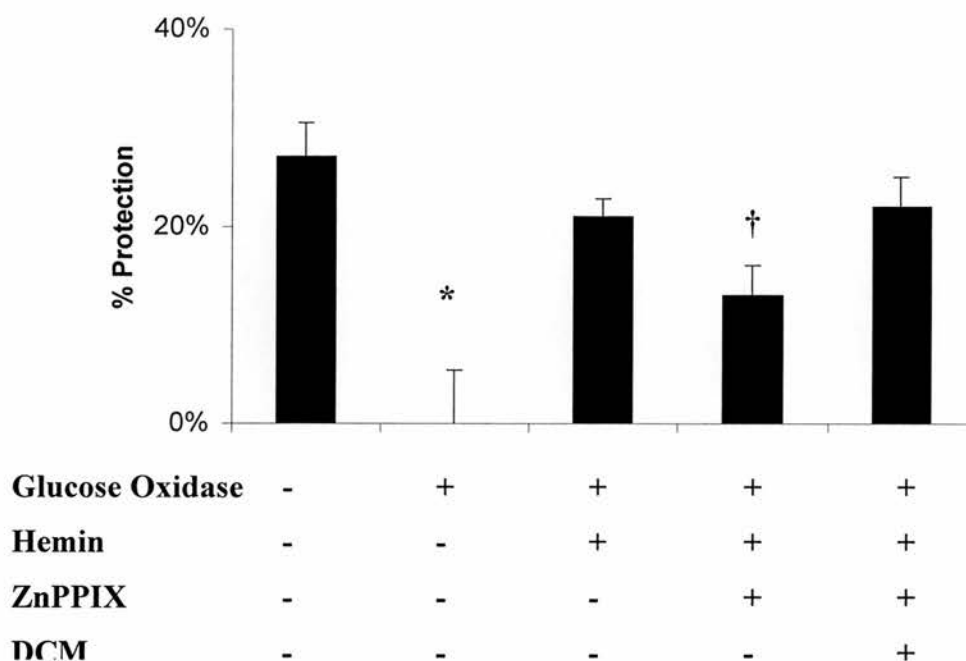


Figure 6.5 Heme oxygenase induction by hemin protects hepatocytes from oxidative injury.

HUH7 Cells were pretreated for 2 hours with 20 μ M hemin, recovered in normal conditions for 4 hours and then challenged with 100mU glucose oxidase. MTT assay was performed 16 hours later. Results are expressed as percentage protection and represent the mean of 3 experiments +/- SEM. The first column represents healthy cells that did not receive glucose oxidase. **ZnPIX** 50 μ M zinc protoporphyrin; **DCM** 0.1% dichloromethane. * = $p < 0.01$ and † = $p < 0.05$ compared with hemin only group.

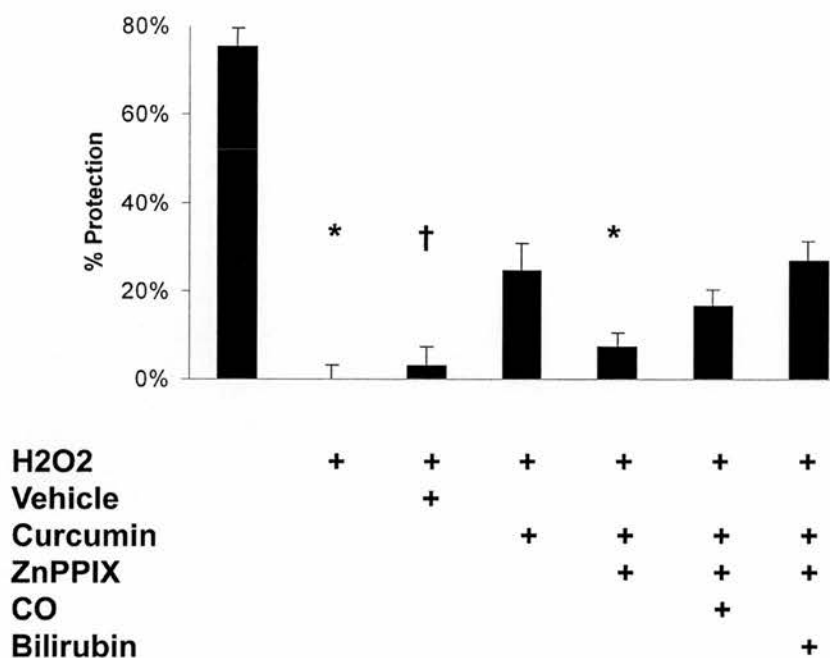


Figure 6.6 Curcumin pretreatment protects hepatocytes from oxidative injury.

HUH7 cells were pretreated for 8 hours with curcumin 10μM or equivalent dose of vehicle, and challenged with 1mM H₂O₂. MTT assay was performed 4 hours later. Results are expressed as percentage protection and represent the mean of 3 experiments ± SEM. The first column represents healthy cells that did not receive hydrogen peroxide. **H₂O₂** Hydrogen Peroxide; **ZnPIX** 50μM zinc protoporphyrin; **CO** 0.1% dichloromethane; **Bilirubin** 1μM bilirubin. * = p<0.01 and † = p<0.05 compared with curcumin only group.

activity to this effect. Administration of zinc protoporphyrin abrogated the cytoprotection seen with curcumin (7 ± 3 , $p<0.01$). Addition of 0.1% dichloromethane partially restored the protection seen prior to HO-1 inhibition (17 ± 4 , $p<0.05$ vs. curcumin and ZnPPiX), demonstrating the protective effect of carbon monoxide generation. Full restoration of protection was provided by addition of $1\mu\text{M}$ bilirubin (27 ± 4 , $p<0.01$ vs. curcumin and ZnPPiX). This was performed three times with similar results.

6.2.4 Development of a model of cold preservation injury

A model of cold preservation injury was developed to mimic the clinical scenario of cold preservation during transplantation. University of Wisconsin solution (UW) is currently the most commonly used preservation fluid in clinical practice in the United Kingdom, and was used for this reason. Cells were pretreated as required and then placed in ice cold UW and stored at 4°C for varying periods of time prior to the UW being replaced with pre-warmed culture medium and being restored to normal culture conditions at 37°C . Lactate dehydrogenase release into the culture medium was used as an outcome marker of cell death.

6.2.5 Curcumin induces HO-1 and is cytoprotective in cold preservation injury

Initial experiments were performed to establish if the HO-1 upregulated by curcumin remained upregulated during the cold storage and reperfusion stage. Time courses performed earlier had revealed that HO-1 was upregulated after 4 to 12 hours of treatment with 10 μ M curcumin (chapter 3). Treatment with curcumin for 6 hours was chosen for further study. HUH7 cells which had been treated with curcumin for 6 hours had their culture medium removed and replaced with UW solution at 4°C, and kept at this temperature for the required period of time, after which they were returned to normal medium and normal culture conditions. Following curcumin treatment, HO-1 was found to remain upregulated during cold storage of up to 48 hours duration. However, after this length of cold storage, there was significant cell death on rewarming, and HO-1 levels dropped dramatically after rewarming. After 16 to 24 hours of cold storage, HO-1 levels remained high following rewarming (figure 6.7). Cold storage for 18 hours was toxic to non-pretreated cells, however curcumin pretreated cells were protected against this toxicity ($p < 0.01$, table 6.1). The degree of toxicity was dependent on the duration of cold storage, and the maximum protection afforded by curcumin pretreatment was found between 16 and 24 hours of cold storage. Greater periods of cold storage removed the protective effect of curcumin (figure 6.8).

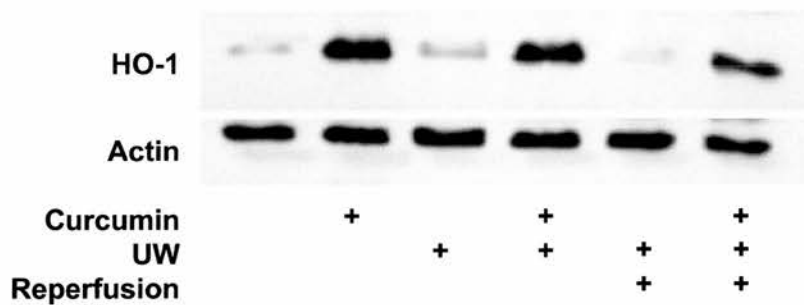


Figure 6.7 Heme Oxygenase 1 remains elevated during cold storage and reperfusion.

Cells were treated as above with curcumin for 6 hours and then incubated in UW solution for 18 hours, followed by reperfusion in normal conditions for 6 hours as indicated. Cell lysates were collected at each stage and a western blot performed for HO-1. Blots shown are representative of three separate repeats.

Replicate	Nil	UW	Curcumin	Curcumin + UW
1	1±0.21	27.92±0.51	1.54±0.14	1.56±0.08*
2	1±0.41	6.91±0.97	1.35±0.62	1.76±1.27*
3	1±0.01	18.61±0.05	1.18±0.04	2.16±0.2*

Table 6.1 Curcumin protects hepatocytes from cold preservation.

Cells were pretreated with 10µM curcumin for 6 hours and then incubated in UW solution at 4°C for 18 hours, and then restored to normal culture conditions for 4 hours, after which the medium was collected and assayed for lactate dehydrogenase activity. Control cells were pretreated for 6 hours. The cells were then cultured in fresh warm medium for 4 hours and the medium collected and assayed for lactate dehydrogenase activity. Data represent three individual experiments, each of which contained 6 repeats within each group, and are shown as mean ± SEM. **Nil** No pretreatment; **UW** cold preservation for 18 hours in UW solution at 4°C; **Curcumin** pretreatment with 10µM curcumin for 6 hours; **Curcumin + UW** pretreatment with curcumin prior to cold preservation. *p<0.01.

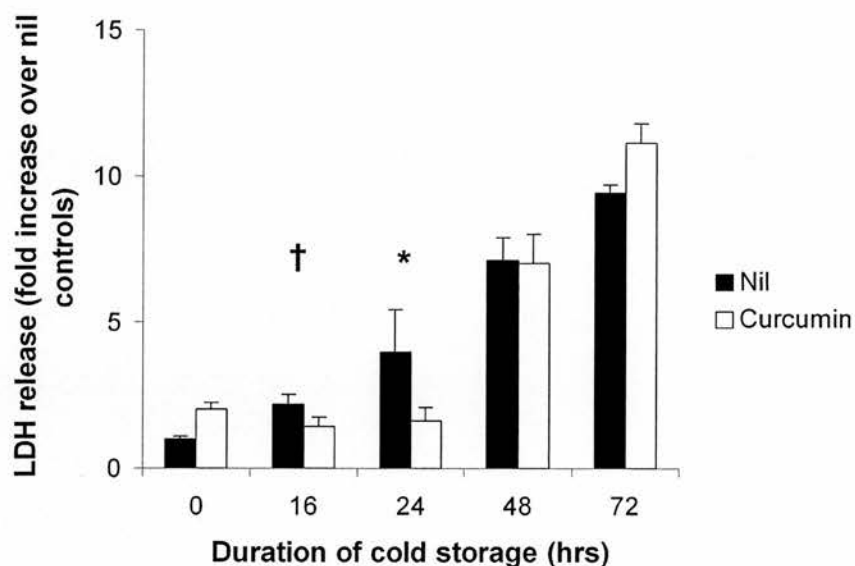


Figure 6.8 Protection from cold preservation is time dependent.

Cells were pretreated with curcumin for 6 hours and then incubated in UW solution at 4°C for a variable length of time, after which they were restored to normal culture conditions for 4 hours. The medium was then collected and assayed for lactate dehydrogenase activity. Results shown are the mean of 6 independent repeats \pm SEM. **Nil** No pretreatment; **Curcumin** pretreatment with 10 μ M curcumin. * = $p<0.01$ and † = $p<0.05$.

6.3 Discussion

6.3.1 Development of *in vitro* models of ischaemia reperfusion injury.

A significant amount of time was invested in developing a robust, reliable and clinically relevant model of cellular injury. There are many such models in the literature, and several were tested before settling on hydrogen peroxide/glucose oxidase as the model of choice.

Sodium arsenite was investigated initially. It is well established as a mediator of oxidative injury and an inducer of the stress response in its own right. It induces ROS production and the MPTP (Petronilli 1994, Kitchin 2001). However, much of its cellular toxicity may be associated with its ability to induce cross-linking of sulfhydryl groups (Petronilli 1994). It therefore does not reflect the events seen in ischaemia-reperfusion injury, and it was a poor choice to model the clinical scenario. Initial results with the model were disappointing. As this was the first model developed, two outcome methods were tried – flow cytometry and MTT assay. However, the results were similar when either method was used. The sodium arsenite was deemed a poor model and abandoned, although the consistency of results between the flow cytometry and the MTT assay suggested that either method could be used with equal validity as an outcome measure in subsequent models.

Anoxia and reperfusion was subsequently studied. Pilot experiments were tested with both primary hepatocytes and HUH7 cells, initially using primary cultures subjected

to 5 hours of anoxia. Previous data in our laboratory predicted that this should be sufficient to cause a measurable degree of cell death. However, no such death occurred. There is always an intrinsic variability in the preparations of isolated hepatocytes obtained from surgical resection specimens, as seen by their variable mRNA responses to a given stimulus (chapter 3). It is likely that the particular preparation of hepatocytes used was more “hardy” than other studied preparations, and so were resistant to the toxic effect of hypoxia. To avoid such variability, HUH7 cells were used subsequently, with a longer duration of hypoxia (16 hours). Again, however, there was little effect on cell viability. Work by others in our laboratory found that HUH7 cells are highly tolerant of anoxia for prolonged periods of time, readily tolerating oxygen deprivation for 24 hours, maintaining metabolism through glycolysis (Ian Currie, personal communication, unpublished results).

Ultraviolet exposure was also used as a model of cellular injury. UVC treatment leads to ROS generation which induces DNA damage, subsequently resulting in apoptosis. Induction of the stress response (by heat) has been shown to be protective against UVC mediated cell death (Chen 1999). It was for this reason that there was some interest in examining the effect of curcumin pretreatment on UVC induced cell death. Initial results were promising and showed that curcumin could protect in this model of cell injury. However, this type of injury is far removed from the clinical scenario of liver surgery. Furthermore, the delayed onset of apoptotic cell death after UVC treatment meant that there was a requirement for a prolonged recovery period before cell death could be assessed. In the setting of HUH7 cells this allows for differences in cell proliferation rates to affect the measured results. Demonstrating

that HO-1 was the protective mechanism in this model would also be difficult.

Despite the protective effect of curcumin being removed by treatment with ZnPPiX, this cannot be definitely concluded to be the result of HO-1 inhibition (and thus the protection cannot be definitely shown to be due to HO-1 induction). Prolonged incubation with ZnPPiX may itself be toxic to some degree as there was an increase in cell death treatment with this inhibitor. It was for these reservations and potential confounding factors that this model was rejected.

Use of glucose oxidase and hydrogen peroxide to generate oxidative injury became the models of choice. These were developed from published literature which had demonstrated that these models were suitable for demonstrating HO-1 mediated cytoprotection (Clark 2000). Glucose oxidase generates hydrogen peroxide enzymatically throughout the period of treatment, rather than just the effect of the initial bolus seen with using hydrogen peroxide directly. However, these models were developed and used in tandem. The main difference between the two models was a very variable response to glucose oxidase treatment. Whilst a fairly consistent proportion of cells would die after treatment with a set dose of hydrogen peroxide, there was marked inter-experiment variability in the extent of injury when using glucose oxidase. The reason for this was unclear. Glucose oxidase enzymatically oxidises glucose, generating hydrogen peroxide. All experiments were performed in DMEM (HUH7s) or William's E medium (primary cultured hepatocytes) which both contain high levels of glucose. Thus, substrate limitation should not be a contributing factor. The enzyme does however, function optimally at 38°C (Gibson 1964). Experiments were performed with medium that had been pre-warmed to 37°C. It is

possible that during the addition of glucose oxidase to the medium and addition of the medium to the cells, with a necessary and slightly variable time spent at room temperature prior to treating the cells, there may have been a fall in temperature, leading to a variation in enzymatic activity between experiments. The consistent results obtained from the hydrogen peroxide model suggest that the variation seen was not the result of differences in the HUH7 cells between experiments. In retrospect, xanthine oxidase may have been a more physiologically relevant agent to use as it is present in and contributes to, the ischaemia-reperfusion injury seen *in vivo*. The results obtained may have been significantly different, as xanthine oxidase will produce superoxide radicals, and the degree of ROS generation could have been limited by lower levels of xanthine *in vitro* than are found *in vivo*.

6.3.2 Preconditioning by curcumin in models of warm and cold injury

Pretreatment with curcumin prior to oxidative injury led to cellular protection. HO-1 induction is known to protect against oxidative stress (Tullius 2002, Clark 2000). To demonstrate that the effect of curcumin was due to increased HO-1 activity, an HO-1 inhibitor was used. This removed the cytoprotective effect, suggesting that it was directly due to heme oxygenase activity. No HO-1 inhibitor is perfect, and ZnPPiX can actually induce HO-1 after prolonged treatment (Yang 2001). However, the short duration of treatment used in these experiments, with application of ZnPPiX only 30 minutes prior to and during treatment with glucose oxidase/hydrogen peroxide, was thought to be unlikely to have such an effect. The addition of carbon monoxide (via dichloromethane) or bilirubin, both end products of HO-1 activity, partially restored

the protective effect of curcumin. The combination of bilirubin and CO may have had additive effects, fully restoring the protection of curcumin, however this was not tested. This provides strong evidence that the predominant effect of curcumin as a cytoprotective agent in the model studied is a result of increased HO-1 activity.

However, it must be considered that the cytoprotective effect could be mediated through a different and parallel mechanism. If HO-1 activity is essential for survival in the model used, then its inhibition will result in a decrease in survival, and provision of its end-products (bilirubin and carbon monoxide) will improve survival. Conceivably, curcumin could improve survival through a completely independent pathway, whereby chemical inhibition of HO-1 would still lead to impaired survival. For example, many different cellular effects have been attributed to curcumin. It does not induce Hsp70 induction in HUH7 cells (chapter 3), but other factors, such as its ability to inhibit NF κ B (Bharti 2003) were not assessed. A further level of proof of the HO-1 dependent nature of curcumin's protective effect could be obtained by demonstrating that curcumin has no protective effect in HO-1 deficient cells, for example using either transgenic hepatocytes or siRNA. However, as it stands, the experimental design used does suggest that the protective effect of curcumin is mediated through HO-1, and is consistent with the increase in HO-1 levels seen with curcumin treatment and the known effects of HO-1 induction by other agents such as hemin (Clark 2000, Motterlini 2000).

Curcumin pretreatment also protected cells from cold preservation injury. Curcumin treatment resulted in upregulation and maintenance of HO-1 levels during cold

preservation and reperfusion, and this was associated with cytoprotection. University of Wisconsin solution was chosen for the model of cold injury because this is the medium in which liver grafts are most commonly stored, and if curcumin was ever to have a clinical role in transplantation, it would have to be able to exert its effect within this environment. Also, UW solution contains many elements such as glutathione which help to minimise the injurious effect of cold storage (Southard 1990). If curcumin was found to be beneficial in the setting of a different solution which did not contain these components, the finding would be of interest, but may be less clinically relevant. It is important to also consider that only cold injury and rewarming occurs in the experimental conditions used. The multiple events seen with *in vivo* reperfusion were not mimicked in any way. It would be possible to perform this experiment with exposure to glucose oxidase during the re-warming phase which would in part address this deficiency.

If curcumin were to be used to precondition human livers for transplantation, it would have to be able to induce HO-1 prior to the organ being harvested. Liver transplantation usually occurs with retrieval of the organ from a brain dead individual (heart beating donor). Curcumin would have to be administered between the time of diagnosis of brain death and the organ retrieval. Despite all efforts to keep this time as short as possible, in clinical practice there are often many hours between the diagnosis of brain death, the gaining of consent for organ donation, and the physical harvesting of the organ. Curcumin requires a minimum of four hours to induce HO-1 in HUH7 cells (chapter 3), and in the experiments in this chapter, 6 hours of pretreatment with curcumin was used prior to cold preservation as this is a

common length of time from consent to donation and the organ being harvested. There is therefore sufficient time for curcumin administration to upregulate HO-1. Although the experiment was not performed, it would be of interest to know if the presence of curcumin during the rewarming period would induce a beneficial effect, as this would be simple to administer in the clinical setting.

Balogun examined curcumin as an inducer of HO-1 in kidney cells, and its ability to protect from cold storage injury (Balogun 2003). Following a three hour incubation with curcumin at 37°C, a stepped reduction in temperature to 20°C and then 10°C for 1.5 hours each was used to enable curcumin to efficiently upregulate HO-1 prior to subjecting the cells to storage at 4°C. This phased cooling was effective, however, it may be difficult to deliver clinically, and maintaining a liver graft at 20°C and then 10°C may lead to harm to the graft that cold storage was designed to eliminate.

Whilst the implementation of curcumin preconditioning in heart beating donors may be difficult, it may be of greater use during living donor donation or in elective liver surgery. In such elective situations, there is ample time to administer a preconditioning agent which may require several hours or even days to have its full effect.

It was also of interest that curcumin lead to protection from cold injury for up to 24 hours duration, but not beyond. Some preconditioning treatments do allow prolonged storage of liver grafts prior to successful reimplantation, even up to 48 hours of storage (Redaelli 2002), which is far in excess of the 12 hour limit currently used in

clinical practice. Curcumin treatment could potentially enable prolongation of cold storage to 24 hours, which may be of use in certain situations where a prolonged period of cold storage is envisaged.

Initial studies to test the efficacy of curcumin as a preconditioning agent in a whole organ would require an animal model. This would be best achieved using a partial hepatic ischaemia model, for example clamping the left lobe for a period of 30 minutes, followed by 24 hours recovery. The effect of curcumin could be established by measuring transaminase release and assessing the degree of histological damage. This would demonstrate protection from ischaemia-reperfusion injury. To confirm if this protection remained in cold ischaemic injury, an ex-vivo reperfusion model could be used, subsequently proceeding to a full liver transplant model. Confirmation of an HO-1 dependent mechanism could be studied with the use of chemical inhibitors of HO-1 (e.g. ZnPPiX), and lentiviral shRNA. Additional confirmation of HO-1 dependency could be achieved by comparison with adenoviral delivered HO-1, and assessment of the ability of carbon monoxide and bilirubin to reproduce the effect in the face of HO-1 inhibition.

Whilst the application of curcumin to animal models would be straightforward, clinical studies would be more challenging primarily due to its poor oral bio-availability and the lack of a parenteral preparation. The significant attraction of curcumin as a therapeutic agent to induce HO-1 is that it is a dietary constituent and is normally taken in up to 1g/day in certain areas (Ammon 1991). Furthermore there have been phase I and II trials of curcumin which have established its tolerability in

high doses, up to 3.6g/day (Sharma 2004), with few side effects other than some mild gastrointestinal upset.

However, when given orally, the absorption of curcumin is poor and the greatest concentration occurs in the bowel epithelium (Garcea 2005), and so the systemic levels reached may be insufficient to induce HO-1. One study which examined the concentration of curcumin following oral administration found that it was present at 2 μ M in the liver tissue, and that it was not present in measurable amounts in serum (Garcea 2004). The results from chapter 3 demonstrate that 10 μ M curcumin is required *in vitro* to induce HO-1. However, this was performed as a single treatment. The effect of regular exposure to 2 μ M may be sufficient to induce HO-1 if given over a period of time. Also, curcumin is rapidly glucuronidated and expelled in the bile. Thus a global measurement of 2 μ M in the liver biopsy may not reflect the level to which hepatocytes themselves were exposed.

Another consideration is that in the liver, hepatocytes do not exist in isolation. Experimental evidence suggests that Kupffer cells play a role in signalling danger to hepatocytes, and hence may play a role in hepatocellular HO-1 regulation (Paxian 2001). It may be that a smaller dose of curcumin reaching the Kupffer cells may trigger intercellular signalling such that hepatocytes are stimulated indirectly to induce HO-1. Whilst this has not been tested in the experiments presented here, this model could be tested *in vitro* in isolated Kupffer cells and hepatocytes, in a variety of co-culture conditions. If Kupffer cells are not stimulated to produce an amplification of the stimulus, oral absorption of curcumin can be increased by

concomitant administration of black pepper (Shoba 1998), which increases bioavailability by 2000% in human subjects. This could potentially provide a hepatic concentration of 40 μ M based on previous studies (Garcea 2004). If sufficient systemic levels could not be achieved, parenteral administration of curcumin could be considered. However, this would require suitable preparation and approval by the Medicines Health Regulatory Authority (MHRA).

Once a method of administration which produced relevant tissue levels of curcumin was established in a phase I trial, its ability to induce HO-1 *in vivo* in humans could be assessed by its administration to patients undergoing elective liver surgery. This will provide a sample of tissue which could be used to assess HO-1 protein expression and activity, and also the effect of systemic administration of curcumin in patients undergoing liver surgery could be assessed. A larger study would be required to demonstrate evidence of a protective effect with HO-1 induction, and this would need to be performed in a group of patients undergoing major liver resection (3 or more segments) with portal clamping (the Pringle manoeuvre) to provide ischaemia-reperfusion injury. Only after this study was completed and found to be safe could curcumin be tried in the setting of human organ transplantation.

6.4 Conclusion

Curcumin robustly induced HO-1 in human hepatocytes, and this was cytoprotective in two *in vitro* models of cellular injury that mimic the clinical scenarios of ischaemia-reperfusion and transplantation. This suggests that curcumin may be a clinically useful preconditioning agent, although more work is required to demonstrate this in a human study.

Chapter 7

Cyclosporin and the Stress Response

7.1 Introduction

The work in previous chapters has examined the effect of curcumin on the stress response and assessed whether it could be used as a preconditioning agent in human hepatocytes. Cyclosporin has been implicated by other groups as a potential preconditioning agent, through its ability to induce the stress response (Yang 2001, Chen 2002), although this has not been established in the human liver. The experiments in this chapter examine the effect of cyclosporin treatment on stress protein expression in human hepatocytes, and whether pretreatment with cyclosporin has a cytoprotective effect.

Cyclosporin A is an immunosuppressant drug commonly used in transplantation. It was purified from the fungus *Tolypocladium inflatum*, and was the mainstay of immunosuppressive therapy for several decades, although in some areas it is now being replaced by newer agents (Regazzi 2005). The effect of cyclosporin on stress protein expression has been examined in the past by other groups. Paslaru's group have found that it induces Hsp27 and Grp78, and this is associated with activation of HSF-1 and HSF-2 (Paslaru 1994 and 2000). It has been found to induce Hsp70 in primary cultures of rat hepatocytes, and to reduce the severity of ischaemia reperfusion injury in a rat liver model (Andres 2000, Saxton 2002). However, its effects have not previously been tested in human hepatocytes.

Aim: To establish the effect of cyclosporin A on the expression of stress proteins in human hepatocytes, and to assess its effectiveness as a pharmacological preconditioning agent.

7.2 Results

7.2.1 Choice of vehicle

Cyclosporin A is highly lipophilic and it is virtually insoluble in water or cell culture medium. Previous groups using cyclosporin have used cremophor, ethanol or dimethylsulfoxide (DMSO) as vehicles. Initial experiments examining the effect of the various vehicles alone demonstrated that cremophor would be unsuitable as it had a degree of toxicity at higher doses, and it also induced a degree of Hsp expression. DMSO and ethanol had no effect in the required concentrations, and it was decided to use ethanol for all subsequent experiments with cyclosporin.

7.2.2 Cyclosporin is toxic in high concentrations

HUH7 cells were treated with increasing concentrations of cyclosporin for 24 hours and then cell survival measured by MTT assay (figure 7.1). There was a dose dependent reduction in survival with increasing doses greater than 10 μ M ($\dagger p < 0.05$).

7.2.3 Heat shock protein expression

The pattern of heat shock protein expression following cyclosporin treatment was examined in HUH7 cells. Initial experiments were guided from the literature, and doses ranging from 0.1 μ M to 50 μ M were used. Cells were treated with cyclosporin and western blots were run for Hsp70 and HO-1.

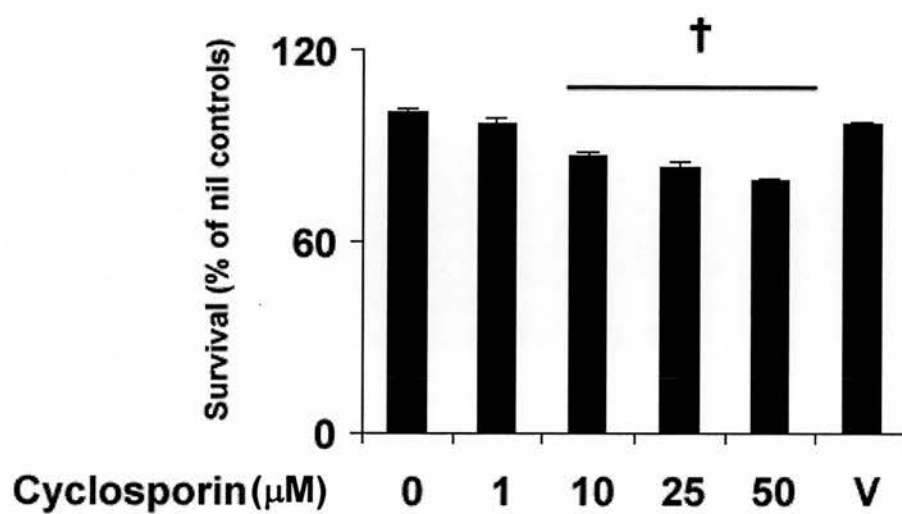


Figure 7.1 Cyclosporin is toxic in high concentrations.

HUH7 cells were treated with increasing doses of cyclosporin for 24 hours and survival measured by MTT. Concentrations of 10μM and greater led to a dose-dependent reduction in survival. †p<0.05.

There was no response in Hsp70 in the range tested, despite the dose response being repeated using a variety of treatment durations. On some occasions Hsp70 was elevated after treatment with 50 μ M, but this effect appeared to be due to a vehicle effect, as the same concentration of ethanol produced a similar induction of Hsp70. HO-1 was found to be induced at 10 to 50 μ M, which was maximal when cells were treated for 24 hours (figure 7.2). This dose responsive induction was consistent within experiments. However, there were often differences between experiments, and on many occasions there was no detectable HO-1 induction.

Further western blots performed on cells treated with cyclosporin at the above doses demonstrated that there was no induction of BiP (Grp78), Grp94, Hsp90, or Hsp27 when treated for up to 24 hours. When repeated in primary cultures of isolated human hepatocytes, induction of HO-1 was variable between preparations, and was induced to a lesser degree than that seen in HUH7 cells. No consistent induction was seen in the expression of any other Hsps examined.

7.2.4 Confirmation of HO-1 induction

Initial western blotting results had demonstrated that HO-1 protein was induced by 10 μ M cyclosporin in HUH7 cells. In order to confirm that this induction was the result of *de novo* synthesis and led to an increase in functional enzymatic activity of HO-1, further experiments were performed to measure activity of an HO-1 reporter encompassing the full length human promoter, to assess any increase in mRNA

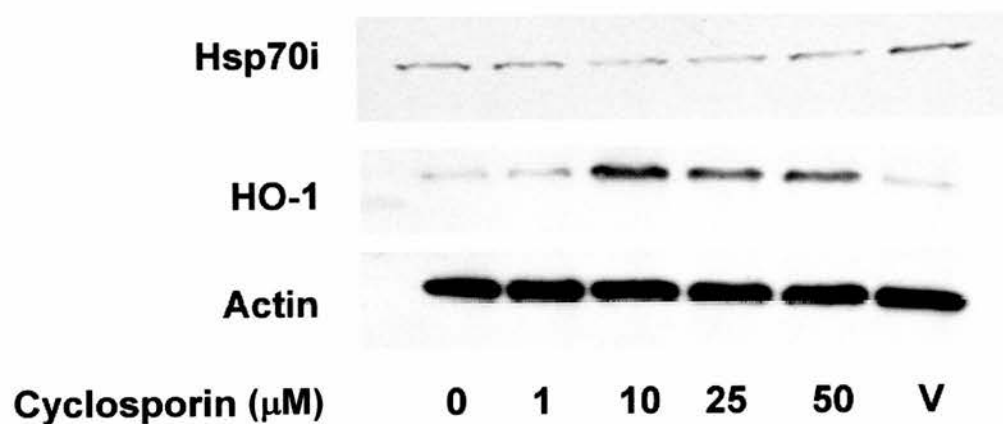


Figure 7.2 Cyclosporin induces HO-1 but not Hsp70 in HUH7 cells.

Cells were treated with increasing doses of cyclosporin for 8 hours as indicated (1-50 μ M), and then western blot performed for Hsp70 and HO-1. V vehicle (ethanol, equivalent of 50 μ M).

levels, and to measure heme oxygenase activity after treatment with 10 μ M cyclosporin.

HO-1 and Hsp70 reporter activity was measured in HUH7 cells (figure 7.3). HO-1 reporter activity was increased after cyclosporin treatment for 21 hours. The induction was modest, with a mean induction of 1.2 fold greater than control (non-significant). Hsp70 reporter activity was reduced following 10-25 μ M cyclosporin treatment ($p<0.05$). Vehicle had no effect on HO-1 reporter activity, but did slightly increase Hsp70 reporter activity (non-significant).

HO-1 mRNA was measured using real time PCR. It was performed on isolated human hepatocytes from three different liver preparations. HO-1 was increased by cyclosporin treatment in a dose responsive manner (figure 7.4). As with the western blotting results, there was a large degree of variability in the results obtained with isolated hepatocytes, although HO-1 mRNA levels were increased in every treatment group. The dose response was performed only three times, which did not permit statistical analysis of the results. Hsp70 mRNA was also measured in isolated human hepatocytes. There was no induction of Hsp70 mRNA, consistent with results from the western blotting.

Heme oxygenase activity was measured in HUH7 cells following treatment with 10 μ M cyclosporin for 24 hours. Total activity was increased greater than two-fold from 141 to 321 pmoles bilirubin/mg protein/hour (* $p<0.01$, figure 7.5). Vehicle had

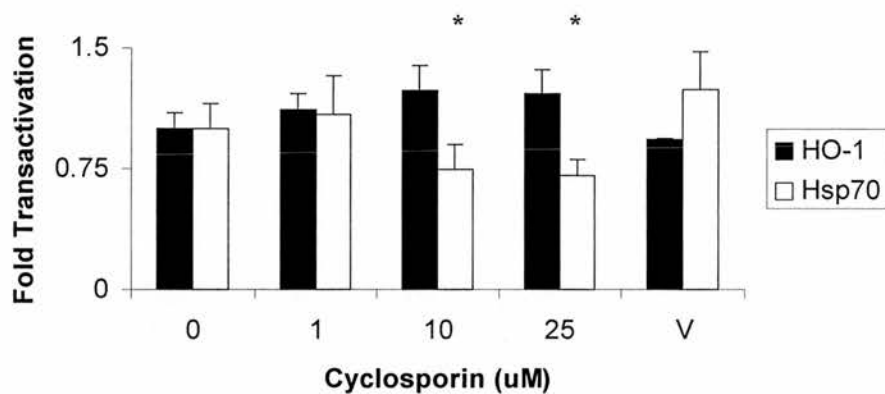


Figure 7.3 Hsp70 and HO-1 reporter activity in HUH7 cells after cyclosporin treatment.

Cells were treated with increasing doses of cyclosporin for 21 hours (**1-25 μ M**) and reporter activity measured. Cyclosporin led to a modest induction of HO-1 reporter activity (non-significant), and reduced Hsp70 reporter activity at doses of 10-25 μ M (*= $p < 0.05$). Results shown are the mean \pm SEM of 6 repeats. **V** vehicle (ethanol equivalent to 10 μ M).

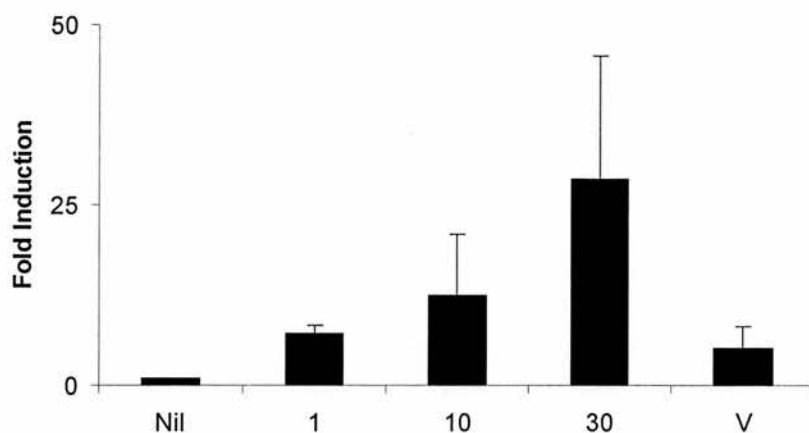


Figure 7.4 HO-1 mRNA levels in isolated human hepatocytes following cyclosporin treatment.

Cells were treated with cyclosporin for 5 hours and then mRNA isolated. HO-1 mRNA levels were measured by real time PCR. Graph shows the mean \pm SEM of three repeats

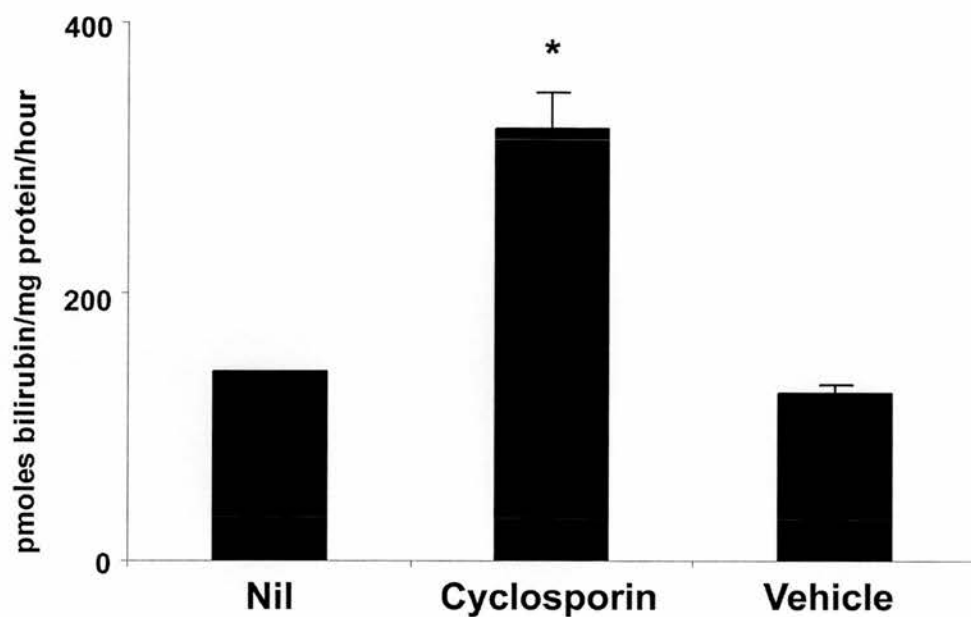


Figure 7.5 Heme oxygenase activity in HUH7 cells after treatment with 10µM cyclosporin for 24 hours.

Cyclosporin treatment significantly increased heme oxygenase activity from 141 to 321 pmoles/bilirubin/mg protein/hour (*= $p < 0.01$). **Vehicle** equivalent ethanol treatment.

no significant effect, lowering the activity slightly to 126 pmoles bilirubin/mg protein/hour.

7.2.5 Localisation of HO-1 expression in Liver Slices

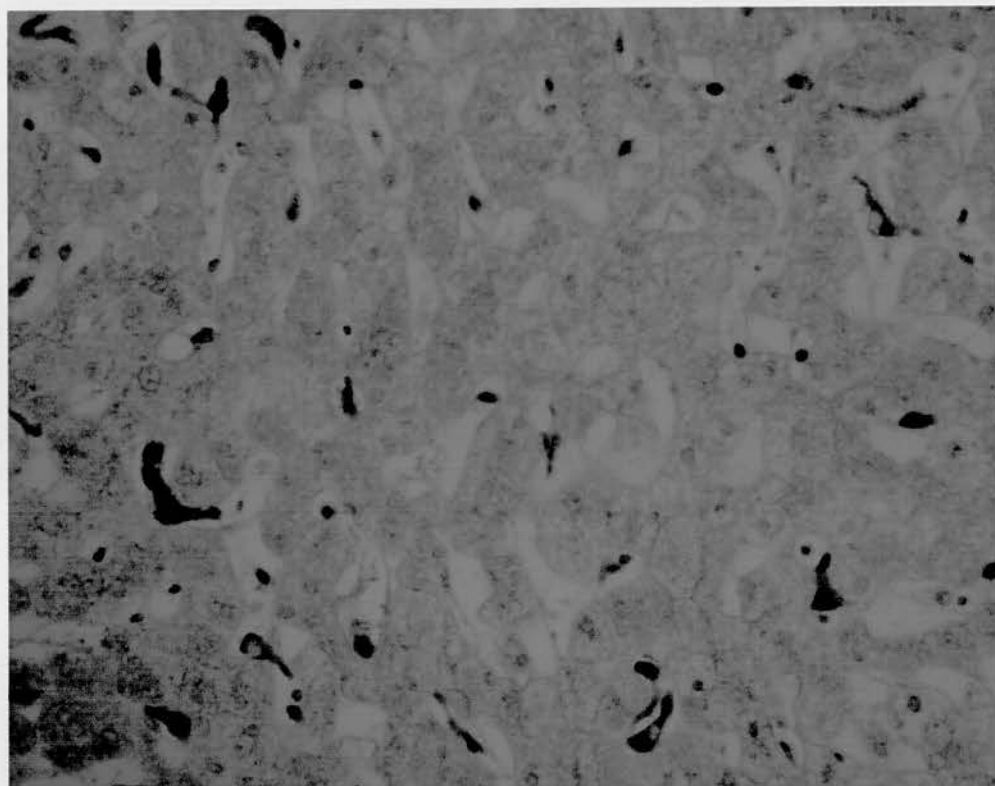
Liver slices were prepared as described in chapter 2, and cultured in the presence of 10 μ M cyclosporin or equivalent vehicle controls. There was a concentration of HO-1 in the Kupffer cells which was seen in control groups and was unchanged by cyclosporin treatment. Hepatocytes demonstrated a low basal expression of HO-1, and this appeared to be unchanged by cyclosporin treatment (figure 7.6).

7.2.6 Effect of Cyclosporin on HSF-1

Although Hsp70 was not induced in hepatocytes treated with cyclosporin, previously published reports from other cell lines had suggested that HSF-1 could be activated by cyclosporin treatment. For this reason, the activation state of HSF-1 was examined following cyclosporin treatment.

It was decided to see if cyclosporin had any effect on HSF-1 when given at 10 μ M, a dose which was sufficient to induce HO-1. HUH7 cells were used, and HSF-1 activation was assessed by native gel electrophoresis to assess trimerisation, gel mobility shift assay (EMSA) to assess DNA binding, and differential electrophoretic mobility on a standard gel to assess hyperphosphorylation (figure 7.7). Cyclosporin

A



B

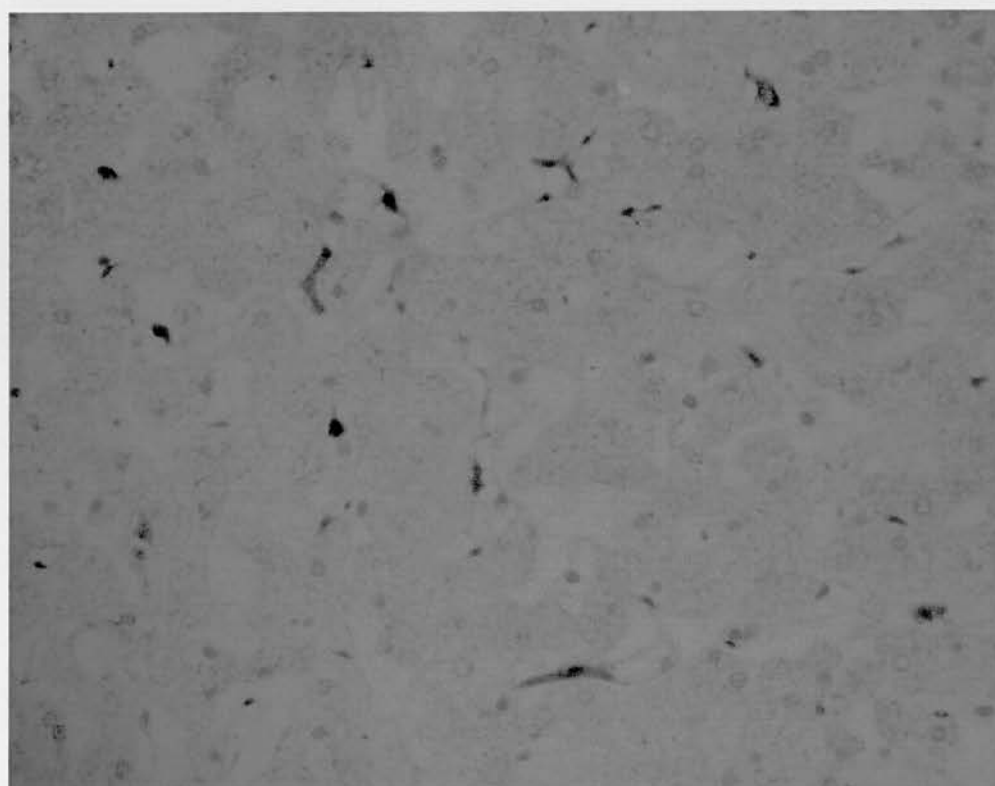


Figure 7.6 Immunohistochemistry for HO-1 in Cultured Liver Slices.

A. Treatment with 10uM cyclosporin A for 6 hours. **B.** Equivalent Ethanol for 6 hours.

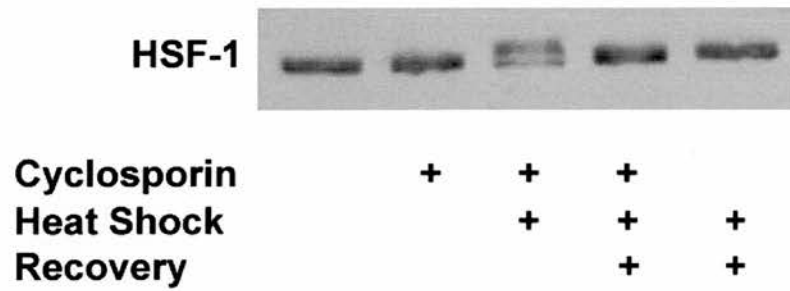


Figure 7.7 Cyclosporin does not activate HSF-1 in HUH7 cells.

Cells were treated with 10 μ M cyclosporin for 2 hours and whole cell extracts were run on a denaturing gel for relative electrophoretic mobility. Heat shock (43°C for 45 minutes) induced a slower mobility band demonstrating hyperphosphorylation of HSF-1. No such band was seen in cells treated with cyclosporin. Cyclosporin also had no effect on prolongation of the period of hyperphosphorylation.

treatment had no discernible effect on HSF-1 activity or status in any of the modalities tested.

7.2.7 Activation of HIF-1 α

HIF-1 α activation can induce HO-1, and for this reason, it was examined by gel mobility shift assay (EMSA) to see if cyclosporin may be inducing HO-1 through HIF-1 α . Cyclosporin treatment was demonstrated to stimulate HIF-1 α binding as measured by EMSA (figure 7.8). This was only evident after treatment with 30 μ M of cyclosporin for two hours.

7.2.8 Cytoprotective Effects of Cyclosporin Treatment

Cyclosporin was assessed as a potential preconditioning agent. Following earlier model development the glucose oxidase model of injury was used (see chapters 2 and 6). Both HUH7 and primary cultures of isolated human hepatocytes were tested. Doses of 0.1 μ M to 10 μ M cyclosporin were used as doses which induced HO-1 without leading to toxicity.

In HUH7 cells, cyclosporin was used on three separate occasions to precondition the cells, with 6 replicates in each treatment group on every occasion. The cells were treated with 0.1 to 10 μ M cyclosporin continuously for 24 hours prior to challenge with glucose oxidase. After treatment with 10 μ M cyclosporin, there was a small increase in survival on one occasion, which was statistically significant (109% vs

100%, $p < 0.05$). However, this was not reproducible on two subsequent repeats (see table 7.1A). There was no effect with doses lower than 10 μ M.

In primary cultures of isolated human hepatocytes, cyclosporin was tested for a preconditioning effect on 6 occasions, with 6 replicates each time treated as above. On two occasions cyclosporin treatment led to an increase in survival, although this was still less than that seen with vehicle treatment (ethanol, see table 7.1B). On the other four occasions there was no protective effect seen with cyclosporin treatment. On one occasion there was an increase in survival with treatment of 0.1 μ M, but this was also less than that seen with the relevant vehicle control.

To explore the possibility that cyclosporin might predispose the cells to protection if a further stimulus was applied, additional heat shock treatment was introduced.

Primary cultures of isolated human hepatocytes were pretreated with 10 μ M cyclosporin for 7 hours before being heat shocked at 43°C for 45 minutes, and then returned to normal conditions in the cyclosporin containing medium for the remaining 16 hours before being treated with glucose oxidase. In two experiments the combination treatment led to a statistically significant increase in survival which was greater than that seen with untreated controls ($p < 0.05$, table 7.1C). However, only one of these was statistically significant when compared with the vehicle control. Four subsequent separate experiments failed to reproduce the protective effect.

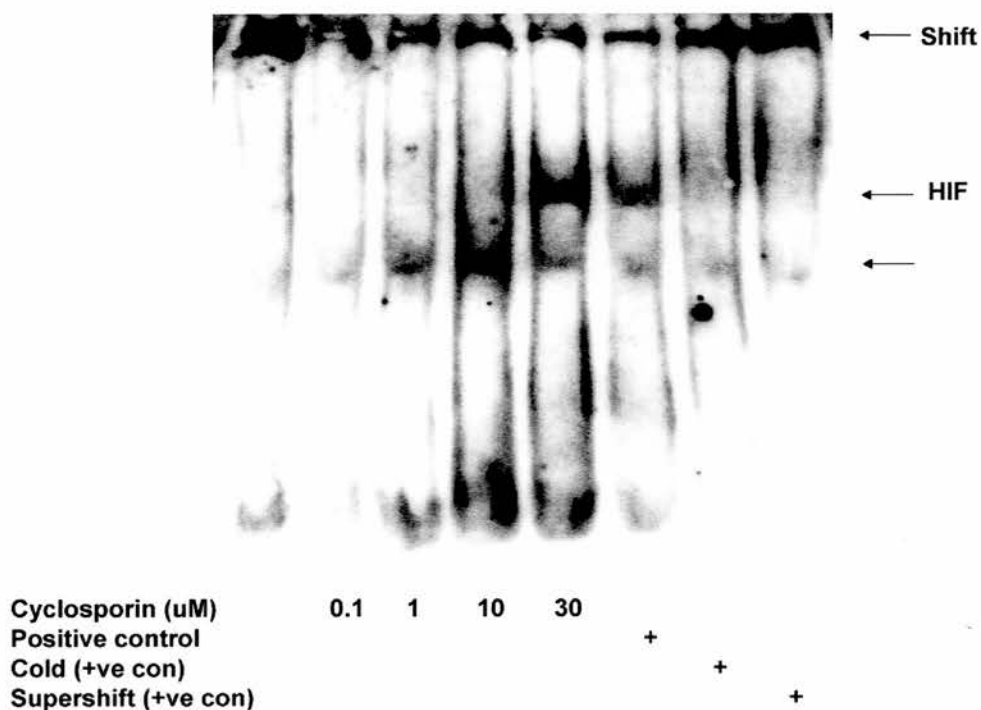


Figure 7.8 HIF-1 α is activated in HUH7 cells by cyclosporin A.

Cells were treated with increasing doses of cyclosporin A for two hours and then nuclear extracts were prepared. Cyclosporin treatment led to the appearance of a lower band at 1 and 10 μ M (unlabelled arrow). After treatment a higher weight band appeared, similar to the band seen in a hypoxic control.

A

	Nil	CsA 10	V
1	100	109 †	101
2	100	75	58
3	100	92	97

B

	Nil	CsA 10	V
1	100	92	77
2	100	310 †	392
3	100	96	113
4	100	119	122
5	100	91	96
6	100	71	93

C

	Nil	CsA 10	V
1	115	146 †	114
2	116	263 †	242
3	109	100	96
4	141	111	212
5	100	61	
6	100	90	102

Table 7.1 Cyclosporin does not precondition human hepatocytes.

HUH7 and primary cultures of isolated human hepatocytes were pretreated with cyclosporin for 24 hours and then cytoprotection assessed in a model of oxidative injury using an MTT assay. Results are displayed as a percentage of non-pretreated cells. † $p < 0.05$ vs Nil control. **A.** HUH7 cells. **B.** Primary cultures. **C.** Primary cultures with additional heat shock (see text).

7.3 Discussion

Initial experiments trialled three different vehicles for diluting cyclosporin.

Cremophor and DMSO had been used in other studies in the literature, however, the use of cremophor led to toxicity in HUH7 cells at the required concentrations, therefore ethanol was chosen as the vehicle for all subsequent experiments. It is possible that a vehicle effect has contributed to some of the stress response inducing effects of cyclosporin in published studies, as not all include appropriate vehicle controls (for example Andres 2000).

Cyclosporin in ethanol was found to be toxic in doses greater than 10 μ M. It is known that some of the deleterious effects of long-term cyclosporin treatment arise from toxic effects, particularly in the kidney. *In vitro* studies have often used doses from 0.1 to 50 μ M, and so these were the doses chosen for further study in the preceding experiments. Andres had found that in primary cultures of rat hepatocytes that cyclosporin was toxic in doses of 10 to 50 μ M after 24 hours of treatment, consistent with the findings in HUH7 cells (Andres 2000).

Confirmation of the actual tissue levels of cyclosporin obtained during treatment is difficult. The original phase I and II trial data remains classified, and Novartis will not release such information at present. Other studies have found that the concentration in the liver in patients with a liver transplant on therapeutic doses of cyclosporin is approximately 3.5 μ g/g of whole tissue (Sandborn 1992). It is difficult to correlate this with the experiments performed here, as the cellular concentration of

cyclosporin was not directly measured. One group has published that uptake of cyclosporin into HepG2 cells (another human hepatocyte cell line) is approximately 50% of available cyclosporin when given in the presence of LDL, as would be the case in the above experiments (Rifai 1996). If this were translatable to the HUH7 cells, this would mean an approximate cellular concentration of 10mg/g, thus far in excess of the levels obtained in clinical practice. Other published studies show plasma concentrations of cyclosporin of 100 to 38000 μ g/l in healthy volunteers (Sgoutas 1986, Gurecki 1985), although levels of around 300 are more common clinically (Sandborn 1992). This gives plasma levels in the range of 0.1 to 32 μ M. Therefore the levels examined in this chapter are for the most part greater than would be seen in clinical practice, although they may still be achievable by oral dosing.

Examination of Hsp expression after cyclosporin treatment yielded variable results. Hsp70 was never induced, and HO-1 was induced on most, but not every occasion in HUH7 cells, and was only minimally induced in primary cultures. The reason for these differences is unclear. During the courses of the experiments several different bottles of cyclosporin were used. Although these were bought from a reputable dealer (Sigma) and of tissue culture grade, it is possible that there was some inter-batch variation in their biological activity. The cyclosporin was always made fresh from a stock stored at the recommended conditions (2-8°C). However the drug may not have been as stable as described, and there may have been degradation of its biological activity during storage. There is also the possibility of contaminants, although the bottles were only opened with careful aseptic technique within tissue culture hoods. There is, as described in earlier chapters, a degree of variation in the

preparations of primary cultures. However, this would not explain the results from the HUH7 cells which should have behaved in an identical fashion in each experiment, thus suggesting that the problem did not lie with the cells themselves. As mentioned above, LDL levels change the cellular uptake of cyclosporin (Rifai 1996), and it may be that there was variation in the lipoprotein content of the fetal calf serum used during the course of the experiments. No experiments were performed in serum free culture.

Although HO-1 was induced to a variable degree, Hsp70 was not induced. This is in contrast to other groups results in cardiomyocytes and in a kidney whole organ model (Chen 2002, Yang 2001). In rat hepatocytes, Andres found an increase in Hsp70 following treatment with 50 μ M cyclosporin for 24hours, which was associated with significant toxicity, with 30% cell death (Andres 2000). This is the same degree of cellular toxicity obtained in HUH7 cells with the same cyclosporin treatment, and it is possible that the difference reflects differences in the cell types' responses to cell death. This is analogous to previous findings of Hsp70 following curcumin treatment, where even toxic doses of curcumin did not lead to Hsp70 induction (chapter 3).

HO-1 was induced by treatment with 10 μ M cyclosporin. This was variable in both HUH7 cells and primary cultures as discussed above. This was reflected in the results for the reporter activity, mRNA levels and HO enzymatic activity, which show variation in the degree of response between the three methods. This likely reflects the differential success in inducing HO-1 protein on different occasions. The

liver slices did not demonstrate a large increase in HO-1 in hepatocytes. However, this was performed using immunohistochemistry and was only used to provide qualitative data on the localisation of HO-1 within the treated liver, where HO-1 was seen to be mostly concentrated within the Kupffer cells, and this was unchanged by cyclosporin treatment.

In view of a previously published report that cyclosporin activates HSF-1 in kidney cells (Paslaru 2000), this was examined in HUH7 cells. There was no activation of HSF-1 in any of the methods tested. There is a concern that, with such apparent variation in the biological effect of cyclosporin between experiments, activation of HSF-1 may have been missed. However, there was at no point induction of Hsp70 with cyclosporin treatment, and when combination treatment of cyclosporin and heat shock was performed, there was also no additional induction of Hsp70 over that seen with heat shock alone. This appears to confirm that HSF-1 was not modified or activated by cyclosporin.

Cyclosporin has been demonstrated by other groups to induce production of reactive oxygen species (Chen 2002), and hypoxia inducible factor 1 α (HIF-1 α) has been shown to respond to chemical oxidative stress in the liver (Tacchini 2002). HIF-1 α is a potent inducer of HO-1, and it was for this reason that HIF-1 α activity was studied. It appeared that cyclosporin treatment did increase HIF-1 α activation as measured by EMSA. Whilst the human HO-1 promoter does contain a consensus binding sequence for HIF-1 α , it has recently been established that hypoxia does not induce HO-1 in human tissues, although it does in other species (Kitamuro 2003). Recent

evidence suggests that in human cells, hypoxia induces the inhibitory factor Bach1, preventing the upregulation of HO-1 by hypoxia. In the absence of hypoxia, however, HIF-1 α could bind to the HRE and mediate HO-1 induction in response to a chemical stimulus. Of note, however HIF-1 α activation did occur at higher treatment doses (30 μ M), which would be consistent with the doses known to induce ROS production (Chen 2002).

As noted earlier, HO-1 induction was seen following treatment with 10 μ M cyclosporin, the level at which toxicity was identified. The toxicity and HO-1 induction could both be related to ROS generation. At this concentration, however, with increased generation of intracellular ROS, many non-HIF pathways could also be activated (e.g. Nrf2 or AP-1). Whilst this was not directly explored because of the inconsistent induction of HO-1, it could have been tested by siRNA or Nrf2 and HIF-1 α as performed in chapter 5. From the data obtained, it is not possible to conclude whether HIF-1 α contributes significantly to HO-1 induction by cyclosporin.

Final experiments regarding cyclosporin involved examining it for a cytoprotective effect. This was assessed in HUH7 cells and in primary cultures of isolated human hepatocytes. Despite initial experiments in each cell type showing a possible protective effect, this was not reproducible. It has to be concluded that there is no preconditioning effect of cyclosporin treatment on human hepatocytes. This conflicts with results from other groups where cyclosporin was able to protect cardiomyocytes from oxidative injury (Chen 2002), and protect kidneys in an ischaemia reperfusion model (Yang 2001). However, as discussed above, both of these studies found that

there was induction of Hsp70, which may account for their cytoprotective phenotype, whilst in contrast, human hepatocytes did not produce Hsp70 following cyclosporin treatment.

7.4 Conclusion

Cyclosporin was not found to consistently induce the stress response in human hepatocytes. It was able to induce HO-1 expression in a very variable manner, and it is unclear why the results between experiments was inconsistent. Cyclosporin was not able to induce preconditioning in *in vitro* models of oxidative injury, and would not be a candidate preconditioning agent.

Chapter 8

Effect of HO-1 Induction on Differentiated Cell Function

8.1 Introduction

Earlier chapters have focussed on chemical induction of HO-1 and the mechanisms through which this occurs. However, whilst there are numerous agents and stimuli which induce HO-1, there has been little examination of the effect of HO-1 induction on other functions of the cell. The HO-1 pathway is of doubtless importance, however it is often thought of as a single separate entity within a cell, and collateral effects both of the various inducing agents and of the products of HO-1 activity are generally not considered. The premise of this work is the development of a preconditioning agent for therapeutic use. It is for this reason that this chapter examines the effect of several different methods of HO-1 induction on differentiated cell function within hepatocytes.

Hepatocytes are the synthetic powerhouse of the liver and the body, producing important proteins such as albumin and fibrinogen. They are also involved in the response of the body to infection or inflammation, producing large volumes of acute phase proteins such as C reactive protein (CRP) and α 1-antichymotrypsin. They are involved in body iron metabolism by synthesis of transferrin, and are also critical in the clearance of waste products such as urea. They have further roles including detoxification of drugs and other agents by the action of various cytochrome oxidases. It is a requirement for any therapeutic manipulation of HO-1 in the liver that it does not impair the many other diverse activities of hepatocytes.

Aim: To examine the effect of HO-1 induction on differentiated cell function in human hepatocytes.

8.2 Results

8.2.1 HUH7 cell line

Initial experiments were performed to determine which acute phase proteins were synthesised by HUH7 cells. They were found to produce undetectable amounts of CRP, haptoglobin or α 1-antichymotrypsin. However, they did generate measurable amounts of transferrin and fibrinogen, and these were chosen for further study. Curcumin (10 μ M) and hemin (20 μ M) were chosen as chemical inducers of HO-1, and the synthesis of fibrinogen and transferrin was then measured in the presence or absence of these agents. Other cells were transfected with a rat HO-1 expression vector (or a control vector) and fibrinogen and transferrin levels measured.

Curcumin suppressed fibrinogen synthesis in resting cells after treatment for 8 or 24 hours (figure 8.1A). It was lowered to 55% of that seen in control cells following 24 hours of treatment (211 ± 13 vs 382 ± 18 in controls, $p < 0.05$). This did not prevent the induction of fibrinogen by the addition of IL-6, although levels in curcumin and IL-6-treated cells were still lower than in cells treated with IL-6 alone (888 ± 14 vs 1073 ± 45 , $p = 0.075$).

Hemin treatment led to a minor reduction in fibrinogen levels to 94% of control levels at 8 hours and 92% at 24 hours, with an identical effect on cells co-treated with IL-6 (92% of controls, not significant). Transfection with the HO-1 expression vector suppressed fibrinogen in resting cells, which was slightly lower than the level

seen in control transfections (245 ± 10 vs 288 ± 24 at 24 hours, $p < 0.05$, figure 8.1A). Transfection with HO-1 also limited the response to IL-6 to a greater degree than the control vector (607 ± 34 vs 795 ± 19 , $p < 0.05$).

Curcumin suppressed transferrin production in resting cells after treatment for 8 or 24 hours (figure 8.1B). It was lowered to 45% and 39% of that seen in control cells following 8 or 24 hours of treatment ($p < 0.05$). IL-6 treatment had a minor effect on transferrin levels, increasing it to 120% of control cells (385 ± 25 vs 318 ± 23). This increase was not seen in curcumin treated cells.

Hemin treatment led to a reduction in transferrin levels to 86% of control levels at 8 hours and 72% at 24 hours. The addition of IL-6 increased transferrin levels to 120% of that in cells receiving hemin alone, but this was still less than that seen in cells treated with IL-6 alone ($p < 0.05$). Transfection with the HO-1 expression vector suppressed transferrin both in resting cells and in cells treated with IL-6. This suppression was greater than that seen in control transfected cells (159 ± 4 vs 252 ± 6 at 24 hours, $p < 0.05$).

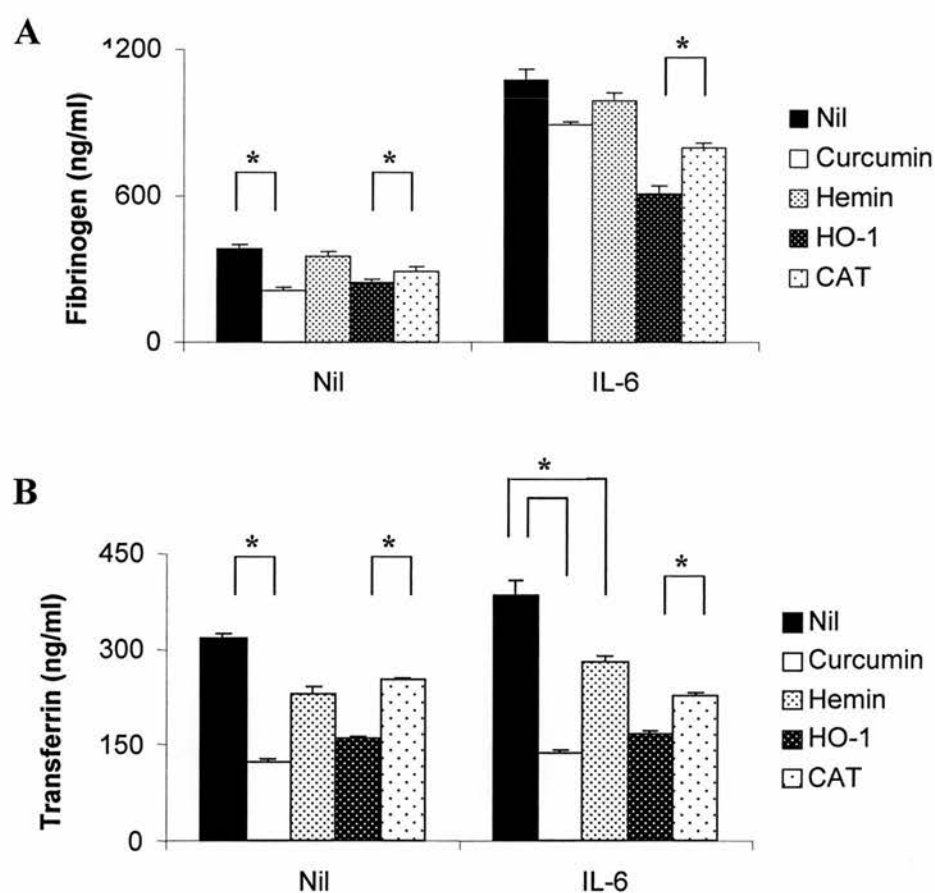


Figure 8.1 Fibrinogen and Transferrin Levels in HUH7 cells following treatment with various HO-1 inducers. A. Fibrinogen B. Transferrin.

HUH7 cells were treated with curcumin (10 μ M) or hemin (20 μ M) or transfected with a rat HO-1 expression vector (HO-1) or control vector (CAT), and the production of fibrinogen and transferrin was measured. The concentration of fibrinogen was measured after 24 hours of treatment, with or without concomitant administration of IL-6. *=p<0.05 between indicated comparison groups.

8.2.2 Isolated Primary Human Hepatocytes

8.2.2.1 Acute Phase Proteins

Fibrinogen, CRP and α 1-antichymotrypsin were chosen as positive acute phase proteins, and transferrin was chosen as a negative acute phase protein. All of these acute phase proteins are synthesised by isolated human hepatocytes. The level of production was then measured after treatment for 24 hours with the HO-1 inducing agents curcumin (10 μ M), cobalt chloride (100 μ M) and hemin (20 μ M). In addition to treatment with these agents, IL-6 and TNF α were used to modulate the acute phase response where indicated.

8.2.2.1.1 Fibrinogen

Fibrinogen levels were unchanged following treatment with curcumin or hemin, whilst cobalt chloride produced a significant reduction compared with that of untreated controls (547 vs 1331 ng/ml, $p < 0.05$, figure 8.2A). Fibrinogen was significantly upregulated by IL-6 (1331 to 3091, $p < 0.05$). There was still a marked response to IL-6 in cells treated with curcumin and hemin, although this was slightly less than that seen in controls (232% in controls, 195% and 162% respectively in curcumin and hemin treated cells). Cobalt chloride completely prevented any response to IL-6. TNF α treatment lowered fibrinogen levels in all groups. This was unaffected by curcumin treatment, but hemin remained capable of inducing fibrinogen in the presence of TNF α (798ng/ml in hemin and TNF α treated vs 588ng/ml in TNF α only controls, $p < 0.05$, figure 8.2A).

8.2.2.1.2 C Reactive Protein

As with fibrinogen, there was no effect of curcumin or hemin treatment on resting CRP levels (figure 8.2B). Cobalt chloride treatment did slightly reduce CRP levels, but this was not significant (125ng/ml vs 94ng/ml, $p=0.173$). In keeping with its status as a positive acute phase protein, there was a marked increase in CRP levels with IL-6 treatment and, as found with fibrinogen, curcumin and hemin treatment did not prevent the response to IL-6, although it was slightly less than that seen in controls (341 and 291ng/ml respectively, compared with 400ng/ml in controls). In cells treated with cobalt chloride, CRP levels were completely unchanged with IL-6 treatment. TNF α had no significant effect on CRP levels.

8.2.2.1.3 α 1-Antichymotrypsin

The levels of α 1-antichymotrypsin (A1AT) were unchanged by treatment with curcumin or hemin, although they were markedly reduced by treatment with cobalt chloride (763 vs 382ng/ml $p<0.05$, figure 8.3A). There was a mild increase in A1AT levels with IL-6, which was unaffected by curcumin treatment, but was prevented in hemin treated cells. TNF α lowered A1AT levels in all groups tested, but there was no difference with the response seen in control, curcumin or hemin treated cells. Cobalt chloride impaired A1AT production, and this was unaffected by cytokine treatment.

8.2.2.1.4 Transferrin

Treatment with curcumin or hemin had no effect on transferrin levels, although cobalt chloride did significantly reduce transferrin (128 vs 237ng/ml, $p<0.05$, figure 8.3B). IL-6 and TNF α both lowered transferrin levels, in all experimental groups

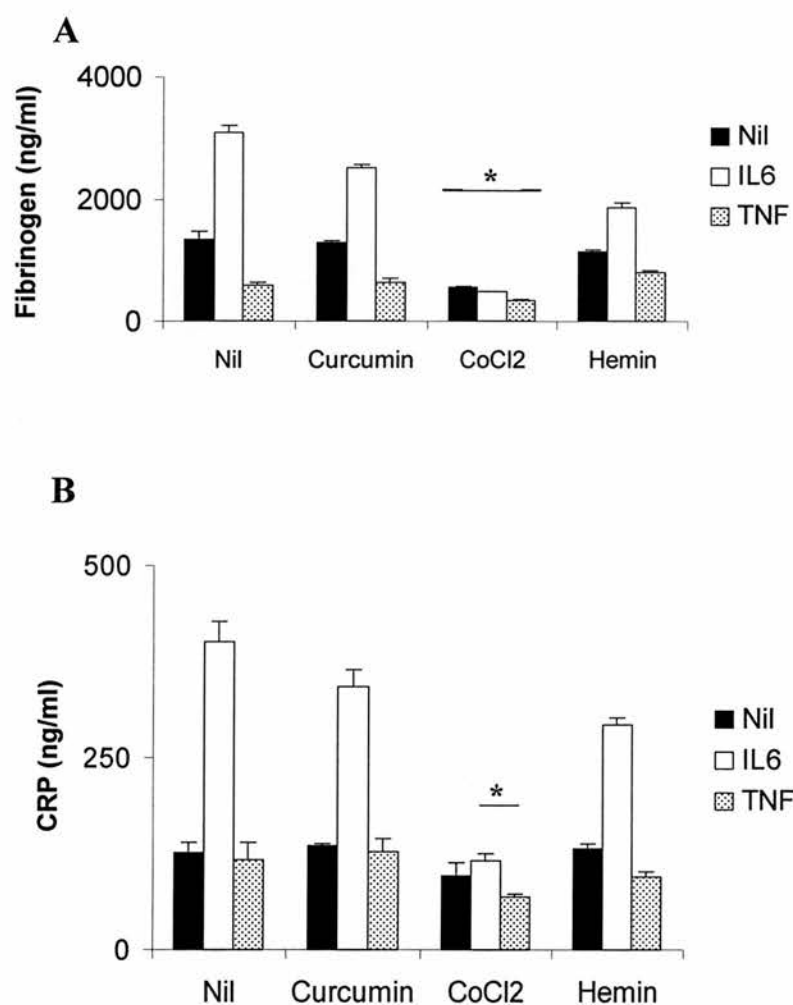


Figure 8.2 Acute Phase Protein Synthesis in Isolated Human Hepatocytes.

A. Fibrinogen. B. CRP.

Isolated human hepatocytes were treated with curcumin (10 μ M), hemin (20 μ M) or cobalt chloride (100 μ M) for 24 hours in the presence or absence of IL-6 and TNF α . Cobalt chloride significantly reduced the expression of fibrinogen and CRP. *=p<0.05 compared with appropriate nil controls.

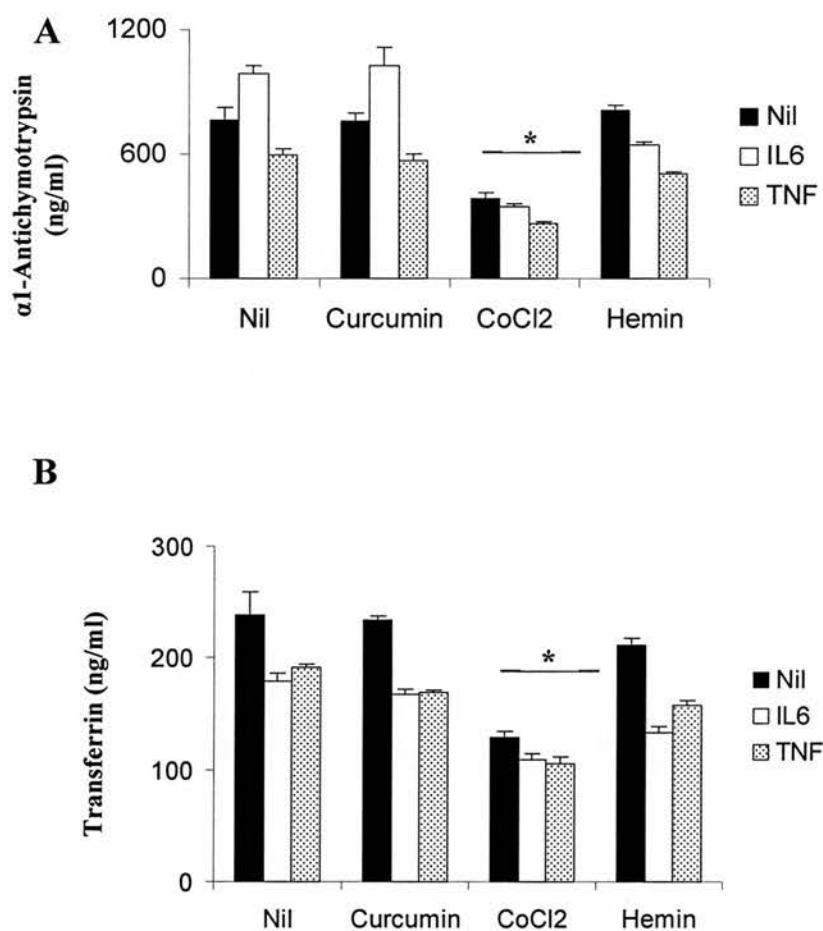


Figure 8.3 Acute Phase Protein Synthesis in Isolated Human Hepatocytes.

A. $\alpha 1$ -Antichymotrypsin. B. Transferrin.

Isolated human hepatocytes were treated with curcumin (10 μ M), hemin (20 μ M) or cobalt chloride (100 μ M) for 24 hours in the presence or absence of IL-6 and TNF α . Cobalt chloride significantly reduced the expression of $\alpha 1$ -antichymotrypsin and transferrin. *= $p < 0.05$ compared with appropriate nil controls.

8.2.2.2 Cytochrome p450

The activities of three cytochrome p450 enzymes were measured: 1A2, 2C9, and 3A4 (figure 8.4). 1A2 activity was significantly increased by curcumin and cobalt chloride treatment (171% and 670% percent of activity in untreated cells, both $p<0.05$). Treatment with hemin or cobalt chloride increased 2C9 activity (202% and 167% of untreated cell, both $p<0.05$). Curcumin marginally increased 2C9 activity, although this was not statistically significant (122%, $p= 0.173$). 3A4 activity was unchanged by curcumin or hemin treatment, but significantly elevated after treatment with cobalt chloride (228% of controls, $p<0.05$).

8.2.2.3 Urea synthesis and ATP levels

Urea production was increased in cells that had been treated with hemin (142% of untreated controls, $p<0.05$) and cobalt chloride (120%, $p<0.05$, figure 8.5). In contrast, curcumin reduced urea synthesis to 49% of untreated controls ($p<0.05$). ATP levels were also measured in hepatocytes following treatment with the HO-1 inducers. There was no change in cellular ATP after any of the treatments.

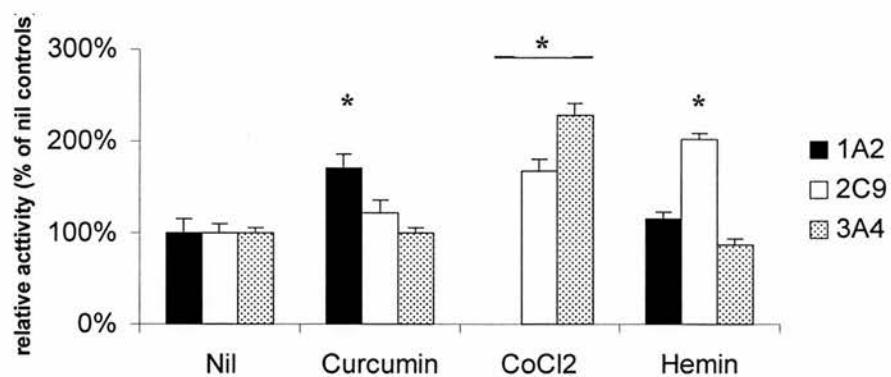


Figure 8.4 Cytochrome p450 activity following treatment with HO-1 inducers.

The activity of the 1A2, 2C9 and 3A4 isoforms of cytochrome p450 were measured in isolated human hepatocytes following treatment with curcumin (10 μ M), hemin (20 μ M) or cobalt chloride (CoCl₂, 100 μ M). Results are expressed as % of untreated controls. The induction of 1A2 activity following cobalt chloride treatment (670% of untreated controls) is not plotted to prevent distortion of the scale. *= $p < 0.05$ compared with appropriate nil controls.

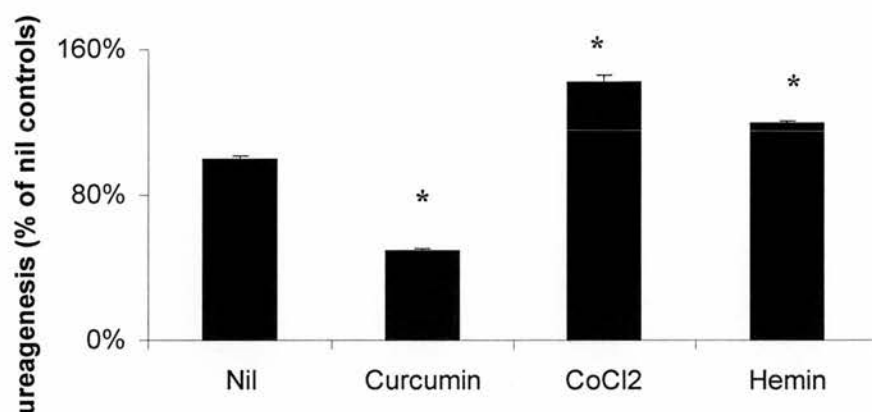


Figure 8.5 Urea synthesis following treatment with various HO-1 inducers.

Urea synthesis was measured in primary cultures of isolated human hepatocytes following treatment with the HO-1 inducers curcumin (10 μ M), hemin (20 μ M) and cobalt chloride (CoCl₂, 100 μ M). Results are shown as % of urea synthesis in nil controls, and represent the mean of 6 repeats \pm SEM. All treatment groups were statistically different from untreated controls, with curcumin reducing ureagenesis and cobalt chloride and hemin increasing it. *= p <0.05 compared with nil control.

8.3 Discussion

There are many different ways to induce HO-1 in hepatocytes. Many of these involve the administration of chemical agents, the full cellular effects of which are unknown. If such strategies are to be used in the liver in clinical practice, it is important to consider the effects of these agents on the various specialised functions of the hepatocyte.

The hepatocyte has many different functions, of which only some were studied in this chapter. The functions chosen were identified as representing clinically important parameters. Acute phase protein synthesis is important in how the body responds to stress and surgery. Cytochrome p450 is important in the metabolism of many drugs, and urea synthesis is the main route of removal of ammonia from the body.

HUH7 cells and primary cultures of isolated human hepatocytes were used in this study. There are significant differences between the two cell types. HUH7s were found not to produce measurable levels of CRP, haptoglobin or α 1-antichymotrypsin. This is consistent with other reports (Nakabayashi 1982). This limited the amount of information which could be obtained from HUH7 cells, and thus primary cultures of hepatocytes were used for further experiments.

The methods of HO-1 induction were treatment with curcumin, hemin or cobalt chloride, or transfection with a rat HO-1 expression vector. The different methods all

induce HO-1 through slightly different mechanisms, and hence may result in different effects on hepatocellular function, with curcumin acting through ROS generation (chapter 5), hemin acting through inhibition of Bach1 (Ogawa 2001) and cobalt chloride stimulating HO-1 through HIF-1 α dependent and independent pathways (Gong 2001). Use of the HO-1 expression vector should have allowed examination of the effect of HO-1 induction in a pure form. However, it was only used in HUH7 cells, as the transfection efficiency obtained in primary cultures was insufficient to allow further study.

Each chemical inducer (hemin, curcumin and cobalt chloride) induces HO-1 at different concentrations. Preliminary experiments confirmed that 20 μ M hemin, 10 μ M curcumin and 100 μ M cobalt chloride produced similar levels of induction of HO-1. Hence, although different treatment doses were used for each chemical agent, they produced similar levels of HO-1. If these agents were to be used clinically for preconditioning, they would each be given in a dose which reliably induced HO-1 and produced the desired cytoprotective effect. Thus, although the dose of each drug used in this study was different, they were selected on the basis that these doses produce comparable HO-1 levels, and their comparison is clinically relevant.

Fibrinogen is a positive acute phase protein, and in both primary hepatocytes and HUH7 cells it was seen to respond appropriately to IL-6. Curcumin and hemin had no effect on either basal or induced levels, whilst cobalt chloride reduced fibrinogen levels. In contrast, in HUH7 cells, fibrinogen levels were suppressed by curcumin treatment, although they still increased with concomitant IL-6 treatment. The reason

for this conflicting result is unclear, but it may represent differences in the HUH7 cells. These are an immortalised cell line, and do not synthesise all acute phase proteins. Hence it is possible that there is aberrant control of acute phase protein synthesis. The primary cultured hepatocytes are much more likely to represent a “normal” response. It is also important to note that there is significantly less fibrinogen synthesised by HUH7 cells than by primary cultured hepatocytes (approximately one third at resting and post IL-6 levels). This is unlikely to be due simply to a difference in the synthetic potential of the two cell types, as the basal level of transferrin in HUH7 cells is greater than that found in primary hepatocytes, and it further implies differential regulation of acute phase protein expression in the two different cell types.

In primary hepatocytes, neither curcumin nor hemin had a deleterious effect on acute phase protein expression, with only slight differences being noted between treated and control cells. Only four such proteins have been assessed here, and only their response to IL-6 and TNF α . However, from the current study there is no evidence to suggest that using these agents to induce HO-1 would significantly alter the synthesis of acute phase proteins if they were used as preconditioning agents in the human body. Cobalt chloride, given at 100 μ M, was found to suppress the production of all acute phase proteins studied.

The synthesis of urea was altered by the HO-1 inducing agents. Cobalt chloride treatment increased ureagenesis, whilst curcumin suppressed it. Hence the suppression of acute phase protein production by cobalt is not simply the result of

generalised inhibition of cellular processes. This demonstrates that various potential preconditioning agents may have different profiles across the different specialist functions of the hepatocyte. For example, cobalt also increased the activity of all cytochrome p450 enzymes studied. Thus cobalt chloride cannot necessarily be seen as an inferior preconditioning agent, as there may potentially be occasions where it would be preferred to have elevated cytochrome activity and a reduced acute phase response. However, from the current results, it appears that hemin was the best agent tested in terms of maintaining normal levels of cellular function.

The cytochrome p450 system is a large family of mixed function oxidases, and are hemoproteins. They are important for the metabolism of a wide variety of drugs and chemicals. One of the reasons for establishing the cytochrome activity following HO-1 induction is that, as they are hemoproteins, they are a potential substrate for HO-1 activity. Whilst some HO-1 inducers such as hemin provide a substrate for the new HO-1 to metabolise, when HO-1 is induced by other agents, such as curcumin, it is unclear which heme groups are degraded by the new formed enzyme. It has been established that the protective effects of HO-1 induction require both substrate and functional activity of the enzyme, and not merely its presence (Foresti 2001). Therefore, in all models where HO-1 has been shown to be cytoprotective, there must be degradation of heme groups. Because the cytochrome p450 system constitutes a significant proportion of the heme groups in hepatocytes, it is of interest to establish the effects of HO-1 induction on this family of enzymes. A study by Yoshida in rat partial hepatectomy noted that there was an inverse relationship between HO-1 and cytochrome p450 during liver regeneration (Yoshida 1984).

However, as shown, with any of the chemical treatments used there was no significant drop in the three cytochromes which were measured. It would have been of interest to examine the effect of the HO-1 expression vector on cytochrome p450 activity. However this was precluded by the low transfection efficiency achieved in primary human hepatocytes.

The cytochrome system is inducible by a variety of stimuli such as hormones, alcohol and bioflavonoid (Waxman 1999). Each enzyme has its own peculiar substrates. The 3A subfamily is one of the most important drug metabolising families in humans. The 3A4 enzyme is important in metabolising a large number of clinically useful drugs including paracetamol and warfarin. Among other substrates, 1A2 metabolises caffeine and 2C9 metabolises non-steroidal anti-inflammatory agents. Thus, there is a significant potential for clinically important interactions if preconditioning agents were to alter the expression of these enzymes.

Curcumin has been reported to inhibit 1A2 in rat hepatocytes (Oetari 1996). This was not found in the current study. Whilst this might reflect species differences, the reason may lie in the different assay methods used. In the method used in this study, cells had been pretreated with curcumin for 24 hours. However, when the protein was extracted from the cells, the actual reaction contained only cellular protein. Curcumin is highly lipophilic, and whilst it may have saturated the cell, it is likely to have been largely removed with the membrane during the protein extraction process. In contrast, in Oetari's study, curcumin was added directly into the assay reaction, and was found to be inhibitory. Thus, whilst Oetari has shown that curcumin can

inhibit the activity of 1A2 when it is present in the chemical reaction, the results of this chapter do not support this as being of relevance to the functional activity of 1A2 within the cellular environment.

It has been shown by another group that curcumin has no effect on the expression of 3A4 (Raucy 2003). This would be consistent with the findings of this study. Of interest, endotoxin suppresses cytochrome p450 levels. Curcumin has been shown to prevent the reduction in 2C11, 3A2 and 2E1 following endotoxin treatment (Cheng 2003). Such a dynamic response has not been studied in this work. However it suggests that curcumin may be a good choice as a preconditioning agent because it may be able to help maintain cytochrome p450 levels.

8.4 Conclusion

Heme oxygenase-1 induction does have an effect on the differentiated cell function of hepatocytes, and this varies between the agents used. Greater understanding of the wider effects of HO-1 inducing agents may enable establishment of scenarios in which distinct agents are chosen in particular circumstances. From the results obtained, hemin was the best agent at maintaining normal hepatocellular function. Curcumin, which has been found in earlier chapters to have preconditioning potential did inhibit ureagenesis, although it had minimal other effects.

Chapter 9

Final Discussion

This work has examined the ability of curcumin and cyclosporin to induce the stress response in human hepatocytes, and the mechanisms through which this may occur. The principle underlying this work is that induction of the cell's natural defence mechanisms in the form of the stress response could be used as a therapeutic strategy to improve outcomes after liver surgery.

9.1 Isolated Human Hepatocytes

This research focused on the study of the stress response in human hepatocytes. Primary cultures of isolated human hepatocytes were prepared from patients undergoing elective liver surgery. There were some limitations on the project as a result of using these cells. The cells were not always available on a regular basis, depending on the scheduling of patients for resectional surgery. This meant that a proportion of experiments were performed in the HUH7 cell line, which allowed guaranteed supply.

There was some intrinsic variability of the primary human hepatocytes between preparations. This is a result of the duration of surgery and other operative factors, but also relates to the age of the patient. Significantly greater yields of cells were obtained from younger patients than from older patients.

One of the other potential areas of criticism of this work is that it relates solely to hepatocytes. In the liver there is a complex arrangement of Kupffer cells, Ito cells

and endothelial cells, which all interact with the hepatocytes. This interaction was not addressed in the current study. Kupffer cells and endothelial cells were independently isolated several times during the preparation of hepatocytes, and the method within the laboratory generated almost pure populations of these cells. Thus, the effect of the interaction of different cell types could be studied using a variety of co-culture models, although time did not allow for this to be performed in the current study.

Slices of liver tissue were prepared and cultured, which could have been used to address some of the effects of cell-cell interaction. However, there were some intrinsic difficulties with this technique. A dedicated tissue slicing machine was not available, and so the slices were cut by hand. This led to minute variations in the thickness of the slices, and this had a profound effect on the viability of the thicker slices. Even in thin slices, there was noted to be patchy cell death and loss of the liver architecture. Thus the culture conditions were not perfect, and although there were several attempts made to optimise conditions, these were unsuccessful. The slices were used in a limited fashion to examine the pattern of HO-1 expression in the liver following curcumin and cyclosporin treatment (chapters 3 and 7 respectively). If the technical difficulties could be overcome, the ability to culture fresh slices of tissue would be of great interest and a valuable laboratory tool.

9.2 Curcumin

Initial experiments examined the bioflavonoid compound curcumin. This natural extract from the plant *curcuma longa* has been used in as a traditional medicine in Indian and Chinese cultures. In human hepatocytes it was found to increase production of heme oxygenase-1, and this was found to be protective in *in vitro* models of hepatocellular injury. Thus, it may have clinical potential as a preconditioning agent.

As described in chapter 4, a considerable amount of time and effort was spent on examining the effect of curcumin on HSF-1. Ultimately this was not rewarded by positive results. The main stimulus for the continued examination of HSF-1 was a combination of the previously published reports that HSF-1 was activated by curcumin in HeLa cells (Dunsmore 2001), and the finding of elevated Hsp70 mRNA levels after curcumin treatment in HUH7 cells (chapter 3). Thus it was important to be certain that an effect on HSF-1 by curcumin had not merely been undetected by the experimental design. It was for this reason that each of the component parts of the HSF-1 activation process was examined.

On reflection it would appear that there are a number of significant differences in HUH7 and HeLa cells. It is apparent from their response to toxic doses of both curcumin and cyclosporin A that HUH7 cells do not respond to such toxicity by producing Hsp70. They do respond appropriately to heat, which does produce HSF-1 activation and Hsp70 as expected. This lack of Hsp70 induction in the face of drug

toxicity is not unique to HUH7 cells, and similar differences in several types of primary and immortalised cell lines in response to curcumin has been demonstrated by other groups (Khar 2001).

Curcumin has many attractions as a preconditioning agent. It is a dietary constituent and is well tolerated when given orally even in high doses, although at very high doses mild gastro-intestinal upset may occur (Garcea 2004). However, as discussed in chapter 6, its bioavailability following oral administration is poor, and may be insufficient to induce HO-1 *in vivo*. Curcumin has been administered parenterally in animal studies and this is a potential route for use in humans, however one of the advantages of curcumin is that it is known to be safe after oral administration. There are no studies in human subjects on parenteral administration of curcumin, and, if this line of treatment were to be pursued, the phase I initial trials and would need to be repeated and the full process of drug development would need to begin from scratch just as for a newly developed agent. This is not insurmountable, but removes some of the attractions of curcumin as a ready to use preconditioning agent.

9.3 Cyclosporin

Cyclosporin was also examined for its preconditioning potential. This was based on reports in the literature suggesting that it was capable of inducing Hsp70 and exerting a preconditioning effect on the kidney (Andres 2000, Yang 2001). However, cyclosporin did not consistently induce the stress response in human hepatocytes in this study. Cyclosporin did induce HO-1 expression in a very variable manner, but it remains unclear why the results between experiments was inconsistent. Cyclosporin showed promise as a preconditioning agent on initial testing (chapter 7). However, this result was not reproducible. A considerable amount of time was exerted in attempting to reproduce the initial results. Ultimately these were not reproducible and thus it has to be concluded that cyclosporin A is not a suitable preconditioning agent.

9.4 Other Preconditioning Agents

Curcumin and cyclosporin A were not the only agents tested. Initial plans were to examine tacrolimus (another calcineurin inhibitor) in addition to cyclosporin A, to look for a class effect of these drugs on the stress response. Unfortunately, the disappointing results with cyclosporin A suggested that tacrolimus may not be a suitable preconditioning agent. A small number of experiments were performed which showed that tacrolimus did induce HO-1. However, by this point the project was fairly advanced and there was insufficient time to explore this agent in detail.

Tacrolimus would be worthy of further study, particularly the mechanism through which it induces HO-1. Indirectly, this work also confirmed that hemin is a good agent to induce HO-1 in human hepatocytes, and that it has a strong preconditioning effect.

9.5 The Ideal Preconditioning Agent

The ideal preconditioning agent would work as a “silver bullet”, having its effect on a specific molecular target, with a specific cellular effect. This would have no unintended effect on other aspects of the cell’s natural functioning. Current understanding of cellular mechanisms and the interplay between different pathways does not provide sufficient detail to be sure from the laboratory that any agent would be entirely specific. There will always have to be a series of experiments to assess the effects of the treatment on the cell as a whole, and then subsequently in whole organ and whole organism treatment.

It is in some ways unlikely that any drug derived from plants or fungi will have a unique effect within the human body. Thus, from a philosophical point of view, it was unlikely that curcumin would work as a “clean” drug, on a specific cell membrane receptor, with no unintentional side effects. The fact that it, in common with several other agents, appears to trigger its induction of HO-1 through generation of ROS demonstrates that many structurally varied compounds can have the same effect on the cell, but this is not the same as a targeted molecular approach. As our

understanding of the interplay between different kinase pathways and transcription factors increases, the goal of developing a single agent to produce a single cellular effect seems in many ways more elusive than ever. However, as technology continues to develop, other approaches and avenues open up. In light of the known cytoprotective effects of HO-1, current understanding of the regulation of HO-1 could be used to develop a novel way of inducing HO-1. The options range from increased substrate delivery in an organ specific manner, through to molecular delivery of transcription factors and ultimately to introducing extrinsic HO-1. Recombinant viral transfer techniques may in time allow the delivery of synthetic HO-1 to specific cells, in a manner that allows the HO-1 to be produced after a certain trigger, and then for it to desist and remove itself after another such stimulus. However, the lack of specificity of current methods should not prevent their use for preconditioning. For example, ischaemic preconditioning is effective and likely harnesses multiple cellular mechanisms.

The initial premise of this work was to assess the ability of the hepatocellular stress response to be pharmacologically induction, thus producing a stress-tolerant phenotype. Since its description over twenty years ago (Tanguay 1983), the stress response has attracted a great deal of research. In this regard, Hsp70 and HO-1 have been identified as potential components of the stress response which could be manipulated for therapeutic use. As this work progressed, it became apparent that curcumin had potent HO-1 inducing properties, with very little effect on HSF-1/Hsp70. Similarly, cyclosporin, although very variable in its effect, also had its main effect on HO-1. Thus, although this work began to examine the stress response

in general, it became increasingly focused on HO-1. The ubiquitous nature of HO-1 and its established ability to protect cells from a wide variety of damaging stimuli mean that it is a good target to seek to modulate for clinical ends. Whilst many agents can induce HO-1, the realistic development of a clinically useful HO-1 inducing agent requires that it be safe, non-toxic, and easily administered with no deleterious effect on the human body. In many ways this work has demonstrated that hemin is a good agent to induce HO-1 in human hepatocytes. It robustly and reproducibly induces HO-1 and results in a tolerant preconditioned phenotype. When compared with curcumin and cobalt chloride, it was the best agent at maintaining normal cellular function whilst still inducing HO-1. Hemin is also physiological, known to be well tolerated and available in a form that has a clinical license for human treatment (as heme arginate for acute porphyria).

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9.6 Practicalities of Therapeutic Preconditioning

The concept of preconditioning, as outlined in chapter one, requires the foreknowledge of an injurious event. Despite this, there are many diverse areas where it could be applied for clinical use. These range across several medical disciplines, but the potential benefit is most apparent in elective surgical procedures which interrupt the blood supply of an organ. It has particular potential for resectional liver surgery and for solid organ transplantation.

9.6.1 Resectional Liver Surgery

In patients undergoing liver resection, there is often a need to clamp the blood inflow to the liver to reduce bleeding during the operation. This is accomplished by the “Pringle” manoeuvre. The vessels are clamped for as short a period as possible to perform the surgery, and then the clamps are removed and the hepatic inflow is restored. This results in an ischaemia-reperfusion injury as described in the introduction. It is apparent that, in this controlled situation, preconditioning treatments would be of benefit to limit the degree of damage sustained during the

ischaemia-reperfusion injury. There is adequate forewarning of the insult which would allow treatment with a preconditioning agent, and an appropriate insult which preconditioning may limit, allowing improved outcomes after liver surgery.

However, current practice is safe and effective, and it is unlikely that preconditioning will be used in straightforward resections where the clamp time is minimal. Indeed, anatomical resections where solely the right or left lobe of the liver is removed require only interruption of the blood supply to that portion of the liver which is being removed, leaving the remaining liver tissue unclamped and unharmed during the operation. Preconditioning is likely to be of most benefit where extended liver resections are to be performed, removing one lobe and a portion the another, which necessitates the use of the Pringle manoeuvre as described, to prevent haemorrhage from the remaining section of liver. This also leaves a smaller amount of functional liver volume postoperatively. One of the limiting factors in larger resections is leaving sufficient working liver tissue, the function of which is often impaired by the ischaemia-reperfusion injury. Preconditioning treatments, in reducing the severity of the insult, will maintain hepatic function to a greater degree, possibly allowing longer clamping periods or extended resections to be performed.

In a clinical trial of ischaemic preconditioning in human liver resection, Clavien found that the improvement in outcomes varied between the population subgroups. In particular, the greatest benefit was seen in fatty livers (Clavien 2003). Although pharmacological therapy may have a different clinical profile, the implication is that preconditioning treatments may be best implemented in selected groups.

9.6.2 Solid Organ Transplantation

Organ transplantation has been one of the great medical successes of our times. It has revolutionised the treatment of organ failure, often allowing the recipient to resume a normal lifestyle (Lazzaretti 2004). Every advance has led to an increased demand for organs for transplantation and despite major improvements in immunosuppression, organ preservation and operative techniques, patients continue to die on waiting lists due to the shortage of donors (UK Transplant 2004). The number of potential recipients in the United Kingdom awaiting organ transplantation has increased annually, reaching more than 7000 by March 2004. New sources of organs, such as living-donors, split livers and non-heart beating donors, has increased available organs to a degree, however, these developments have failed to keep pace with the decline in the available number of cadaveric organs. Novel therapies such as stem cell technology and bio-artificial livers hold promise for the future, but have not yet met their potential. There remains, therefore, a requirement for solid organ transplantation that is unlikely to recede in the near future.

Solid organ transplantation, with its attendant ischaemia-reperfusion injury, represents an area where pre-conditioning of the donor or the donated organs could make a great contribution (McLaren 2003, Kosieradzki 2002), potentially reducing primary non-function and early graft failure, and thus the requirement for re-transplantation. This may allow organs that would currently be discarded to be used, thus expanding the organ pool. However, there are practical and ethical issues

involved in implementing pre-conditioning strategies that are particular to solid organ transplantation.

9.6.3 Administration

Pharmacological pre-conditioning requires administration of a drug. In the case of elective, living donor transplantation, there is adequate planning and time to permit treatment of almost unlimited duration prior to surgery. However, in the case of cadaveric donors, there is a limited window of treatment between diagnosis of brain stem death and the organ harvest. This is of necessity kept to a minimum to maintain the viability of the transplanted organs. Thus, whilst practically the treatment would be in addition to a range of infusions which the donor would already be receiving and would make little difference to the medical management of the donor, the agent would need to be effective within a limited time frame.

This does, however raise some ethical issues. The optimum management of heart-beating organ donors currently involves the administration of a variety of drugs and infusions to maintain the organs in a suitable condition for transplantation. Pre-conditioning agents would aim to improve the target organs, rather than merely limiting the detrimental effects of brain death, thus crossing the boundary from maintenance to treatment, a cause of ethical concern. There are several precedents for this type of active treatment. Hormonal resuscitation with triiodothyronine, vasopressin and methylprednisolone is administered to heart transplant donors (Rosendale 2003), with the aim of correcting the endocrine imbalances that occur

after brain death. These are active treatments, intended to increase the number of organs suitable for transplantation. However, they differ from pre-conditioning in that they represent maintenance rather than therapeutic measures. Thus the boundary between donor maintenance, optimization and pre-conditioning can be considered indistinct.

There is an alternative argument that pre-conditioning may represent part of the normal process of organ harvesting as it has the intention of increasing the chances of a successful outcome and therefore should not require further assent or lack of objection beyond that associated with organ donation itself. In his treatise on the ethics of organ donation, Price suggests that “where an individual has requested that his/her organs be used for transplantation after death, it seems correct to infer that permission is granted for procedures which form part of the routine preliminaries to transplantation without seriously compromising the patient in any way” (Price 2000).

Even where there is no explicit authorisation of procedures for maintaining donors and their organs, they are not necessarily unlawful. However, while explicit legislative provision to cover organ-pre-conditioning techniques may be desirable, it is far more important that society endorses the concept that pre-conditioning strategies in donors to improve organ function and outcome are ethically acceptable and clinically warranted. With regard to organ procurement for transplantation, the UK Human Tissue Bill currently being considered by Parliament states that “it shall be lawful....to take steps for the purpose of preserving that part [part of a body] for use in transplantation, and to retain the body for that purpose”. However, this

authorisation “shall only extend to the taking of the minimum steps necessary....and to the use of the least invasive procedure” (UK Human Tissue Bill 2004). It is unclear whether such authorisation would extend to pre-conditioning techniques.

It is conceivable that the relative importance ascribed to such considerations might alter depending on the nature of the pre-conditioning strategy considered. For example it seems unlikely that many relatives would object to the deceased donor being given a drug to improve organ function but it is possible that they might raise objection if it were necessary to keep the donor ventilated for a prolonged period of time to allow the drug to take effect.

9.6.4 Multi-organ donors

The majority of organ donors in the UK donate more than one organ. This raises the question that if a pre-conditioning strategy had been demonstrated to confer a beneficial outcome for a particular organ, would it be acceptable to use such a treatment in a multi-organ donor if the effects on other organs were not known? The answer in this case would probably be negative and so research involved in the development of such strategies should consider effects on other organs that might be donated simultaneously in a clinical setting. If the effects of a treatment on other organs were not adverse, the question then arises as to whether the potential recipients of other organs should be consulted and requested to give their consent to pre-conditioning being used. The issues of consent and collateral involvement

become more prescient in the context of a clinical trial where the effect of a pre-conditioning strategy may be the subject of evaluation.

9.6.5 Recipients of pre-conditioned organs

In many circumstances where pre-conditioning is used it may be impractical to obtain consent from the recipients in time to influence the application of the pre-conditioning intervention. There is concern therefore that if a recipient objects to receiving an organ which has undergone pre-conditioning this may subvert the normal informed consent process by placing coercion on the recipient. Thus the desire to receive a transplant may outweigh the concerns or objections of the recipient to receiving an organ from a research protocol. It could be argued that pre-conditioning has the potential to improve the outcomes of transplantation and that society should support research which aims to advance science or outcome in medicine. Following this argument to its practical conclusion, the occasional refusal to receive a pre-conditioned organ would be offset by the perceived benefit to society as a whole. In practice perhaps the easiest way to deal with this issue would be to inform patients, at the time of addition to the waiting list, that they may be offered organs derived from trials of pre-conditioning protocols and that they should consider whether they would accept such an organ. They would also be informed that such protocols would have received approval from local regional ethics committees and that such approval would not normally have been granted if there were issues over either safety or perceived benefit.

9.6.6 Pre-conditioning in a research setting

In common with other medical research, trials of pre-conditioning should abide by the principles enshrined within the Declaration of Helsinki (World Medical Organisation 1996). Specifically there should be an adequate laboratory and animal research base to support human studies and these studies should have clear potential for benefit counterbalanced against the inherent risk of the intervention. The design of studies should be clearly set out and be undertaken by competent individuals. Studies should have been reviewed and approved by an independent committee – in the UK this would be the local or multi-centre research ethics committee as appropriate.

Of importance is also the data from Azoulay's study on ischaemic preconditioning in human liver transplantation from cadaveric donors (Azoulay 2005). This found that, although biochemical markers of hepatocellular damage (i.e. serum transaminase levels) were lower following transplantation of a preconditioned graft, initial poor function was more common in these livers. This suggests that although the cells were protected from the transplant-associated injury, the preconditioning technique impaired hepatocellular function. This underlines the importance of understanding the manifold unintended effects which may be seen with such a non-specific treatment. In regard of the pharmacological agents tested in this work, this was explored in chapter 8. All preconditioning agents will need to pass through a rigorous testing procedure before they can be adopted as routine clinical tools. This is of particular importance in the setting of organ transplantation where many parties are

involved and there is a requirement to make the best use of a limited resource. This may therefore limit the testing of preconditioning strategies in the transplantation setting, despite its tremendous potential.

9.7 Future Work

Building on this work, future directions include further development of curcumin and hemin as preconditioning treatments, and exploration of the role that HO-1 plays in the survival of cancer cells.

Due to its aforementioned low bioavailability, curcumin may have limited use as an oral agent for hepatic preconditioning. However, this has yet to be tested, and the attractions of a ready to use, well tolerated agent with few side effects mandates that this be assessed. This could be accomplished simply and easily in a limited study as performed by Garcea, using a small number of patients due for elective liver resection, and measuring HO-1 levels in the resected specimen (Garcea 2004).

It is possible that curcumin will not be suitable as an oral agent. It has been used parenterally in animals, and this requires further study. None of the studies published using curcumin in this way have yet looked at HO-1 expression and its preconditioning potential. Furthermore, derivatives of curcumin such as tetrahydrocurcumin may have greater biological activity than curcumin itself (Okada 2001). This has yet to be assessed in terms of its HO-1 inducing potential. Also, the

interplay between different cell types may be important, and there may be an amplification of HO-1 induction by hepatocytes in the presence of Kupffer cells. This could be studied simply *in vitro* using co-culture models.

One of the outcomes of this work was that hemin was found to be a very clean inducer of HO-1, with minimal effects on the other functions of the hepatocyte. The availability of hemin in a form for parenteral administration (heme arginate – licensed for use in porphyria), makes this a attractive prospect for further study. Heme arginate should be tested *in vitro* to confirm that this form is effective as a preconditioning treatment, and then appropriate animal and human studies could be performed depending on results.

In a different direction, the findings of chapter 9 demonstrated the contribution that HO-1 genotype makes to outcomes of two malignancies. It would be of interest to pursue this along several different avenues. Confirmation that tumours with the SS genotype actually do manufacture greater levels of HO-1 than LL genotype cancers would add to current knowledge. If this was confirmed, it raises the possibility of developing anti-HO-1 therapies to help treat cancer, the premise for which has already been established (Fang 2003). Of more relevance to liver surgery, now that the technique is established, the effect of HO-1 genotype on liver transplantation can now be studied.

9.8 Conclusion

This work has added to the understanding of HO-1 induction and the cellular mechanisms through which curcumin is effective. It has demonstrated that curcumin induces HO-1 in human hepatocytes and that this occurs through oxidant pathways. It also established that curcumin is a candidate preconditioning agent for human hepatocytes. It does, however, have an effect on other important functions of the hepatocyte. It merits further study, and clinical phase I/II trials are warranted.

Cyclosporin was not found to be a useful preconditioning agent, and it was extremely inconsistent in its effect. Hemin, however, as a reliable agent to induce HO-1 which has minimal other effects on the cell, may in due course be established as a clinically useful agent for pharmacological preconditioning.

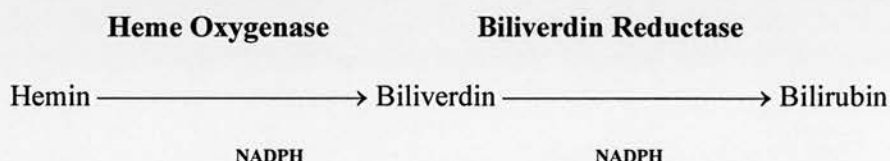
Preconditioning as a concept is now established. It holds great potential for a wide sphere of clinical scenarios, and heme oxygenase-1 appears to be an ideal molecule to exploit to achieve preconditioning. It only remains for the ideal therapeutic agent to be established to permit preconditioning to be used as a clinical tool which may in the future become part of everyday best practice.

Appendices

Appendix A

Analysis of the Effects of Different Concentrations of Heme on Apparent Heme Oxygenase Activity

The end products of heme oxygenase are carbon monoxide, biliverdin and free iron, all of which can be measured. Monitoring carbon monoxide production requires specialist equipment (Marks 2002). For this reason, many researchers use a combination paired enzyme reaction with biliverdin reductase, first described by Tenhunen in 1968 (Tenhunen 1968):



Bilirubin levels are then measured by a standard spectrophotometric method using the difference in absorption at 454 and 530nm ($\epsilon = 40\text{mM}^{-1}\text{cm}^{-1}$). This technique has been performed in several different ways over the past 30 years (Tenhunen 1968 and 1972, Motterlini 1996, Cuturi 1999 and Chauveau 2002) and appears to be robust. There is a degree of variation in the way the assay is performed by different laboratories. For this reason, the methods were compared in detail, and the effect of different protein concentrations and heme concentrations were compared.

The basic reaction components do not vary between methods described in the literature. All contain: hemin as a substrate for the reaction; an NADPH generating system; a source of biliverdin reductase; and the experimental tissue sample or cell lysate. The reaction is performed at 37°C and pH 7.4. Whilst the method for purifying biliverdin reductase from fresh rat or human liver is well established (Kutty 1981, Yamaguchi 1994), the use of an unprocessed 105,000g supernatant from homogenised liver obviates the requirement for prolonged purification steps which

remove over 60% of biliverdin reductase activity. Attempts within this laboratory to purify biliverdin reductase from human liver lysates resulted only in the production of a very dilute extract with no discernable enzymatic activity. This loss of activity was noted during the precipitation stage. Thenceforth, unprocessed 105,000g supernatant was used.

The bilirubin concentration can be measured either directly in the aqueous reaction, or following chloroform extraction. The peak absorbance of hemin and bilirubin is 385nm and 454nm respectively. However, the absorption spectra overlap particularly at the concentrations present in the assay (figure B.1). This results in increasing concentrations of hemin reducing the sensitivity of bilirubin measurement when measured in the aqueous phase (figure B.2).

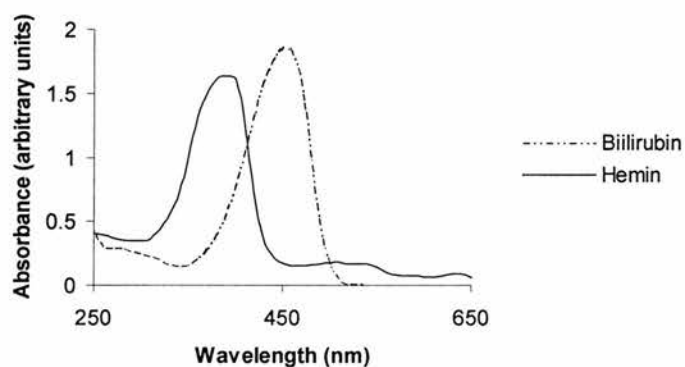
Chloroform extraction ameliorates the reduction in sensitivity seen with increasing hemin concentration. Bilirubin has a low solubility in phosphate buffer at pH 7.4, with a tendency to exist as a colloidal sol or form large flocculants and its true solubility is reportedly less than 0.1 μ M (Lee 1976). The solubility of bilirubin in chloroform is much greater. It is reportedly up to 10mg/ml however, at this concentration, it exists as a particulate suspension. Chloroform can extract bilirubin from an aqueous solution to a maximum of 35 μ M. Whilst hemin is also soluble in chloroform, the extraction from an aqueous phase is much less efficient than with bilirubin. Hence chloroform extraction has the two-fold benefit of solubilising all bilirubin within the reaction, and partially reducing the dampening effect seen with the presence of hemin (figure B.2).

Lower concentrations of hemin will ensure more accurate measurement of bilirubin. However, reducing the substrate concentration affects the efficiency of the enzymatic reaction, resulting in a limitation of bilirubin (figure B.3). Furthermore, large amounts of protein in the reaction inhibit the function of both heme oxygenase and biliverdin reductase (Tenhunen 1972). This was seen with the use of greater than 1mg of protein.

The final method used was as follows: Tissue samples were prepared by homogenisation in buffer (50mM Tris, 20mM NaCl, 10mM KCl, 0.1mM DTT, 1mM EDTA, 1% SDS, pH 7.4), and cultured cells were prepared by collecting in phosphate buffer (0.1M potassium phosphate, 2mM MgCl₂, pH 7.4), followed by three freeze-thaw cycles using liquid nitrogen. The coupled enzyme reaction method was performed in a 400µl mixture containing: 500µg of sample protein; 50µM hemin; 0.8mM NADPH; 2mM G6P; 0.2 units G6P dehydrogenase; and 1mg of liver cytosol (105,000g supernatant). Liver supernatant was prepared fresh, from liver that had been snap frozen in liquid nitrogen, by homogenisation in 0.1M sodium citrate buffer, pH5, containing 10% glycerol. Large volumes of liver cytosol were prepared to minimise variation due to differences in biliverdin reductase activity within preparations from different liver samples. The reaction was prepared in 0.1M potassium phosphate buffer with 2mM MgCl₂, pH 7.4, and a mastermix is used to minimise pipetting error. Following a 1 hour incubation in the dark at 37°C, the reaction is terminated with the addition of 400µl chloroform and the bilirubin generated is measured in a spectrophotometer at A₄₆₀-A₅₃₀ ($\epsilon = 40\text{mM}^{-1}\text{cm}^{-1}$). A

control reaction containing all components except the test protein extract is used for the spectrophotometric blank. Results are expressed as pmoles bilirubin/mg protein/hour. 50 μ M hemin and 500 μ g of protein. The inter and the intra assay coefficient of variation at the concentrations used are both 8%.

A



B

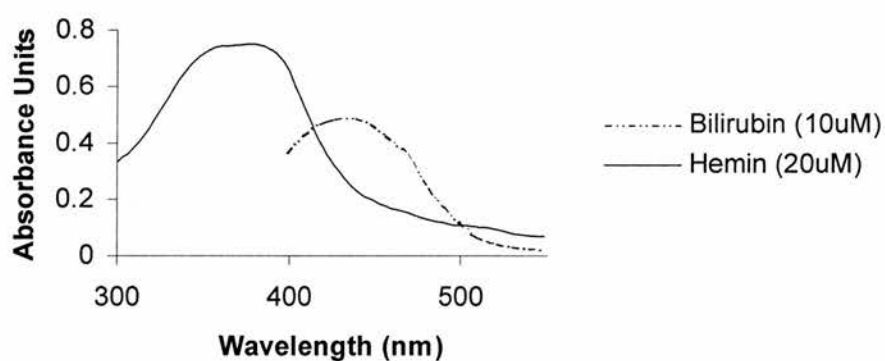


Figure B.1. The absorbance spectra of hemin and bilirubin overlap.

A. Absorbance spectra of hemin in an aqueous solution pH7.4 and bilirubin in chloroform. **B.** Absorbance spectra of 20 μ M hemin and 10 μ M bilirubin in 0.1M phosphate buffer pH 7.4, concentrations commonly seen with the HO assay, showing degree of overlap relevant when measuring A_{460} - A_{530} .

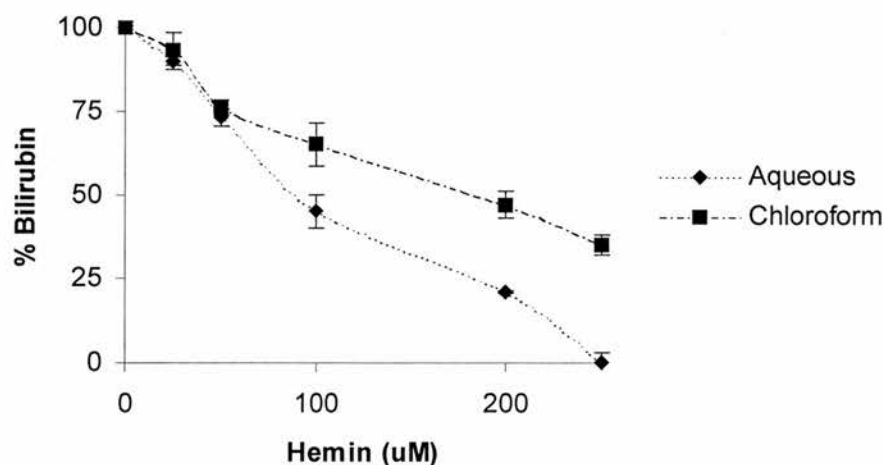


Figure B.2. Hemin reduces the sensitivity of bilirubin measurement.

An aqueous solution of $10\mu\text{M}$ bilirubin was prepared and increasing concentrations of hemin added. There is a stepwise reduction in the apparent bilirubin content with increasing hemin beyond $25\mu\text{M}$.

Chloroform extraction was performed and the extracted bilirubin measured, demonstrating that chloroform extraction lessens the desensitising effect of hemin when present at greater than $50\mu\text{M}$. Results represent the mean $\pm\text{SEM}$ of 6 repeats.

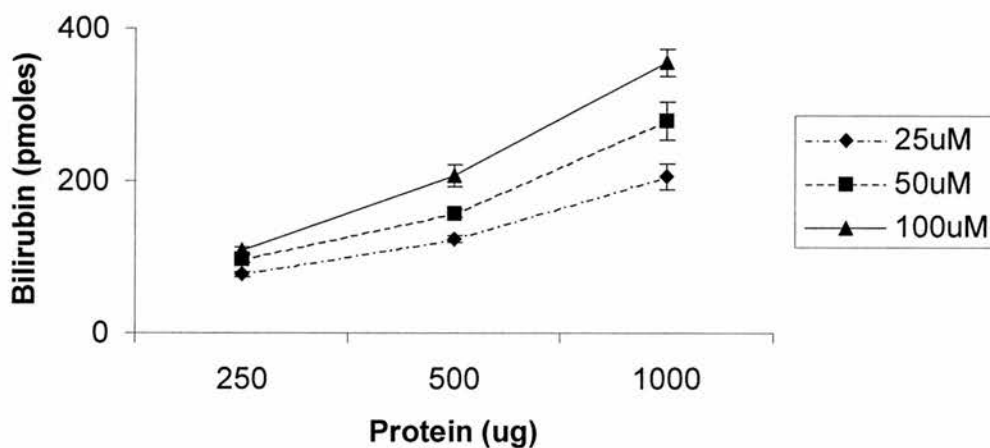


Figure B.3. Hemin concentration affects apparent HO activity. Protein prepared from HUH7 hepatoma cells was used for assaying HO activity. 250 to 1000 μ g of protein was incubated in reactions with varying hemin concentrations. Low hemin concentrations (25 μ M) reduced the apparent HO activity as measured by bilirubin production. A linear relationship of HO activity to protein content was seen when using 250 to 1000 μ g of protein. Results represent the mean \pm SEM of 6 repeats.

Appendix B

Buffers and Reagents

Primary Hepatocyte Isolation

Buffer A	1x HBSS 10mM Hepes
Buffer B	1x HBSS 0.5mM EGTA
Buffer C	1x HBSS
Buffer D	1x HBSS 5mM CaCl ₂ 0.05% collagenase 0.017% hyaluronidase 0.002% DNase

Percoll Gradient

Percoll (Sigma)	90mls
10x HBSS	10mls
Phosphate Buffer pH 7.4	2mls

Liver Perfusion Phosphate Buffer

Na ₂ HPO ₄	2.4g
KH ₂ PO ₄	0.4g
200mls	

HBSS (10X)	Gibco/Invitrogen Cat no 14185-045
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MTT Assay

MTT solution	5mg/ml in PBS
MTT Extraction Buffer	10% SDS, pH 3

LDH Assay

Tris buffer	1M Tris-HCl, pH 8.5
Substrate	0.1mM l-lactate, pH5.5
Chromogen	5mg/ml NAD
	2mg/ml INT
	0.5mg/ml PMS
Stop solution	100µl of 0.5M HCl
Blank solution	0.01M sodium oxalate

Cell Lysis Buffers

Whole Cell Extracts

Protease inhibitors (Protease cocktail inhibitor tablets, Roche)

One tablet/10mls RIPA

Nuclear and Cytoplasmic Extracts

Buffer E	20mM Hepes (pH 7.9)
	10mM KCl
	1mM EDTA
	1mM EDTA
	1mM DTT
	0.1mM Na ₃ VO ₄
	10% glycerol
	0.2% NP40
	Protease inhibitors one tablet/10mls

Buffer F	20mM Hepes (pH7.9)
	350mM NaCl
	10mM KCl
	1mM EDTA
	1mM DTT
	0.1mM Na ₃ VO ₄
	20% glycerol
	Protease inhibitors one tablet/10mls

Native Lysis Buffer

HEPES	20mM
NaCl	350mM
KCL	10mM
EDTA	1mM
DTT	1mM
MgCl	1.5mM
Protease inhibitors	1 tablet/10mls

RIPA Buffer

1x PBS
1% NP40
0.5% Sodium deoxycholate
0.1% SDS

General Buffers

40x TAE	(for 1 litre)	Tris Base	193.6g
		NaAcetate.3H ₂ O	108.9g
		Disodiumdihydrate EDTA	15.2g
		pH 7.2 (with acetic acid)	
10x TBE	(for 1 litre)	Tris Base	107.8g
		Disodiumdihydrate EDTA	7.4g
		pH 8.3 (with boric acid)	
1x TBS (immunostaining)		50mM Tris	
		150mM NaCl	
		pH 7.4	
1x TBS (Western blotting)		20mM Tris	
		150mM NaCl	
		pH 7.4	
PBS		9.1mM dibasic sodium phosphate	
		1.7mM monobasic sodium phosphate	
		150mM sodium chloride	
		pH 7.4	
Western Loading Buffer		3.2ml dH ₂ O	
		5ml 0.5M Tris HCl (pH 6.8)	
		4ml Glycerol	
		8ml 10% SDS	
		2ml Mercaptoethanol	
		1ml 0.05% bromophenol blue	
		added to samples at ¼ volume of sample	

Running Buffer	0.025mM Tris
	0.2M Glycine
	0.0035M SDS

Transfer Buffer (for 5 litres) 15.15g Tris
72g Glycine

10% Polyacrylamide Gel (Western)

Water	4.05ml
1.5M TrisHCl, pH 8.8	2.5ml
10% SDS	100µl
Acrylamide/Bisacrylamide (30% stock, 29:1 ratio)	3.3ml
10% ammonium persulphate	100µl
TEMED	10µl

Stacking Gel (Western)

Water	6.1ml
0.5M TrisHCl, pH 6.8	2.5ml
10% SDS	100µl
Acrylamide/Bisacrylamide (30% stock, 29:1 ratio)	1.3ml
10% ammonium persulphate	100µl
TEMED	20µl

12% Polyacrylamide Gel (GT Repeats)

Water	4.9ml
10x TBE	1ml
Acrylamide/Bisacrylamide (30% stock, 29:1 ratio)	4ml
10% ammonium persulphate	100µl
TEMED	10µl

Reverse Transcription Master Mix

Mg ²⁺	4μl
RT 10x buffer	2μl
dNTPs	2μl
Random primers	1μl
AMV (25u/μl)	1.5μl
RNAsin	0.5μl

Heme Oxygenase Activity Assay Buffers

Buffer G	100mM potassium phosphate
	2mM MgCl ₂
	pH 7.4

Buffer H	0.1M sodium citrate
	10% glycerol
	pH 5

Phosphatase Activity Assay Buffers

Sephadex G-25 Storage Buffer

Tris HCl	10mM
EDTA	1mM
Sodium Azide	0.02%

Phosphatase Storage Buffer

Tris HCl	50mM
EDTA	0.1mM
EGTA	0.1mM
Triton X-100	0.5%
DTT	1mM
β -Mercaptoethanol	0.1%
Protease inhibitors	1 tablet/10mls
pH	7.5

Tyrosine Phosphatase/PP2A 5x Buffer

Imidazole	250mM
EGTA	1mM
β -Mercaptoethanol	0.1%
BSA	0.5mg/ml

PP2B 5x Buffer

Imidazole	250mM
EGTA	1mM
β -Mercaptoethanol	0.1%
MgCl ₂	50mM
NiCl ₂	5mM
Calmodulin	250 μ g/ml

PP2C 5x Buffer

Imidazole	250mM
EGTA	1mM
β -Mercaptoethanol	0.1%
BSA	0.5mg/ml
MgCl ₂	25mM

ELISA Buffers

Coating buffer	0.01M Phosphate Buffer 0.15M NaCl pH 7.2
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Washing/Dilution Buffer	0.01M Phosphate Buffer 0.5M NaCl 0.1% Tween 20 pH 7.2
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Chromogenic Substrate	4x 2mg OPD tablets 12mls H ₂ O 5 μ l of 30% H ₂ O ₂
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0.01M Phosphate Buffer	NaH ₂ PO ₄ .H ₂ O	0.35g
	Na ₂ HPO ₄ .2H ₂ O	1.34g
	In 1 litre of distilled water, pH 7.2	

Antibodies for ELISAs

All antibodies purchased from DAKO. All conjugated antibodies with a PE code were custom made for the laboratory by DAKO and are not in the general catalog.

α 1-antichymotrypsin	Coating	A0022
	Detection (HRP conj)	PE870
CRP	Coating	A0073,
	Detection (HRP conj)	P0227
Fibrinogen	Coating	A0245
	Detection (HRP conj)	P0246
Transferrin	Coating	A0061
	Detection (HRP conj)	PE563

Standard curve made with Human Serum Protein Calibrator (HSPC) X0908 for α 1-antichymotrypsin, fibrinogen and transferrin. CRP standard curve made with human serum CRP calibrator X0923.

P450 Assay Buffers and Conditions

1M KPO₄ (for 100mls) Potassium phosphate, dibasic, anhydrous 13.94g
Potassium phosphate, monobasic, anhydrous 2.72g
pH 7.4 with KOH or H₃PO₄

2x NADPH Generating System NADP 2.6mM
Glucose-6-phosphate 6.6mM
G6P dehydrogenase 0.4u/ml
MgCl₂ 6.6mM
(For 3A4 isoform, also contains: KPO₄ 400mM)

4x Substrate Reaction Mixture

	1A2	2C9	3A4
1M KPO ₄	30μl	0	0
Luciferin Substrate	8μl ME	8μl H	4μl BE
Water	62μl	92μl	96μl

Reaction Conditions

1A2	40 mins	room temperature
2C9	60mins	37°C
3A4	60mins	room temperature

Primers

Real Time Primers

Hsp70

Forward primer	¹ CCTAAGGCTTTCCTCTTGCAAA ²²
Reverse primer	¹ CAGAGTGGCTGCCAAAACTC ²¹
FAM labelled probe	¹ CTGGAGGCCCATGTCTTCCATGTGA ²⁵

HO-1

Forward primer	¹ AGGGTGATAGAAGAGGCCAAG ²¹
Reverse primer	¹ CAGCTCCTGCAACTCCTCAA ²⁰
FAM labelled probe	¹ TGCGTTCCTGCTCAACATCCAGCT ²⁴

HO-1 (GT)_n polymorphism primers

F 5' – GAGCCTGCAGCTTCTCAGA - 3'

F 5' – TCTGGCCATAGGACTTT – 3'

Cytochrome B Primers

F 5' – GGTTCTGCAATAAGAATATAGG – 3'

R 5' – GACAACACAGTAAGAACCAGG – 3'

Gel Shift Oligonucleotide Sequences

HIF-1 α (Santa Cruz – sc2625 and 2626)

Consensus Binding Sequence

F 5' - TCTGTACGTGACCACACTCACCTC - 3'

R 5' - GAGGTGAGTGTGGTCACGTACAGA - 3'

Mutant Oligonucleotide

F 5' – TCTGTAAAAGACCACACTCACCTG – 3'

R 5' – GAGGTGAGTGTGGTCTTTTACAGA – 3'

Nrf2 (Santa Cruz –sc-2527 and 2528)

Consensus Binding Sequence

F 5' – TGGGGAACCTGTGCTGAGTCACTGGAG – 3'

R 5' – CTCCAGTGA^CCTCAGCACAGGTTCCCCA – 3'

Mutant Oligonucleotide

F 5' – TGGGGAACCTGTGCTAGGTCACTGGAG – 3'

R 5' – CTCCAGTGACCTAGCACAGGTTCCCCA – 3'

HSF-1

Consensus Binding Sequence

F 5' - GATCTCGGCTGGAATATTCCCGACCTGGCAGCCGA - 3'

R 5' – TCGGCTGCCAGGTCGGGAATATTCCAGCCGAGATC – 3'

Mutant Oligonucleotide

F 5' – GATCTCGGCTTCAATATTGTCCACCTGGCAGCCGA – 3'

R 5' – TCGGCTGCCAGGTGGACAATATTCCAGCCGAGATC – 3'

Antibodies

Hsp70i (SPA-810)	Stressgen
Hsp70c (N27F34)	Stressgen
Hsp90 (SPA-830)	Stressgen
Grp94 (SPA-850)	Stressgen
Grp78 (SPA-826)	Stressgen
Grp75 (SPA-825)	Stressgen
Hsp27 (SPA-800)	Stressgen
HO-1 (SPA-896)	Stressgen
HO-2 (OSA-200)	Stressgen
HSF-1 (SPA-901)	Stressgen
Actin (sc-8432)	Santa Cruz
Actin (sc-1615)	Santa Cruz
HIF-1 α (sc-13515)	Santa Cruz
Lamin (sc-6215)	Santa Cruz
Nrf2 (sc-13032)	Santa Cruz
phospho p38 (9211)	Cell Signaling
Total p38 (9212)	Cell Signaling

Chemicals and Reagents

Actinomycin D (A9415)	Sigma
Bilirubin (B4126)	Sigma
Catalase (C9322)	Sigma
CoCl ₂ (232696)	Aldrich (Sigma)
Collagenase (LS004196)	Worthington
CCCP (21855)	Fluka (Sigma)
Curcumin (C1386)	Sigma
Cyclohexamide (C1988)	Sigma
Cyclosporine A (C1832)	Sigma
Dichloromethane (D7566)	Sigma
Dihydroethidium (D7008)	Sigma
DAB (diamino benzidine)	Sigma
DPX mountant (44581)	Fluka (Sigma)
ECL (RPN 2108)	Amersham Biosciences
Fugene	Roche
GF109203X (B6292)	Sigma
Glucose -6-Phosphate (G7879)	Sigma
G6P dehydrogenase (G4134)	Sigma
Glucose oxidase (G7141)	Sigma
H ₂ DCFDA (D399)	Molecular Probes
Hemin (H5533)	Sigma
Hyaluronidase (LS002592)	Worthington
INT (I8377)	Sigma
LY294002 (L9908)	Sigma
Mitotracker Red (M7513)	Molecular Probes
Mitotracker Green (M7514)	Molecular Probes
MTT (M2003)	Sigma
N-Acetyl Cysteine (A9165)	Sigma
NADH (N8129)	Sigma
NADPH (N1630)	Sigma

NAD oxidised form	Fluka (Sigma)
Penicillin G (P3032)	Sigma
Phenazine Methosulphate (P9625)	Sigma
OPD tablets (s2045)	DAKO
Percoll (77237)	Sigma
PD98059 (P215)	Sigma
SB203580 (S8307)	Sigma
Sephadex G25	Pharmacia
Staurosporine (S 5921)	Sigma
Sodium arsenite (35000)	Sigma
streptavidin biotin-HRP complex	DAKO
Streptomycin (85886)	Fluka (Sigma)
α -Tocopherol (T3251)	Sigma
Trizol	Invitrogen
Trypsin	Gibco
ZnPIX (282820)	Aldrich (Sigma)

Where not otherwise specified, all chemicals were purchased from Sigma and were of cell culture or molecular biology grade.

Kits

Annexin V-FITC Apoptosis Detection Kit (556570)	BD Pharmingen
Apo-ONE Homogenous Caspase 3/7 Assay (G7790)	Promega
ATP Assay Kit (119107)	Calbiochem
β -Galactosidase Enzyme Assay System (E2000)	Promega
BioRad DC Protein Assay Kit (500-0122)	BioRad
DIG Gel Shift Kit (1 635 352)	Roche
Luciferase Assay System (E4030)	Promega
P450-Glo Assays (V8771, V8791, V8801)	Promega
Serine/Threonine Phosphatase Assay System (V2460)	Promega
Tyrosine Phosphatase Assay System (V2471)	Promega
Urea Nitrogen Kit (no longer available)	Sigma
Wizard Genomic DNA extraction Kit (A1125)	Promega
siRNA Reagents HIF1 α (sc-35661)	Santa Cruz
Nrf2 (sc-37030)	Santa Cruz
Control sequence (sc-37007)	Santa Cruz

Molecular weight markers

Low Molecular Weight Markers	Amersham
High Molecular Weight Markers	Amersham
Non-denatured Protein Molecular Weight Marker Kit (MW-ND-500)	Sigma

Media and Plastics

Cell Culture Plates	Corning
William's E medium (22551)	Gibco
DMEM (41966)	Gibco
University of Wisconsin Solution - Belzer UW solution	Dupont Pharmaceuticals
Fetal Calf Serum	Gibco

DNA/RNA Reagents and Enzymes

RNAse free water (P1193)	Promega
RQ1 DNase (M6101)	Promega
AMV reverse transcriptase (M5101)	Promega
Taq polymerase (M1665)	Promega
dNTPs (U1420)	Promega
25bp, PCR markers, 1kb markers	Promega
10bp DNA Ladder (10821-015)	Invitrogen
Taqman universal PCR mastermix (4304437)	Applied Biosystems

Suppliers and Stockists

Amersham and Pharmacia products ordered from Amersham:

Amersham plc, Amersham Place, Little Chalfont, Buckinghamshire, HP7 9NA, UK.

Applied Biosystems, Division Headquarters, 850 Lincoln Centre Drive, Foster City, CA 94404, USA.

BD Biosciences (BD Pharmingen): BD, 21 Between Towns Road, Cowley, Oxford, OX4 3LY, UK

Cell Signaling Technology, Inc., 3 Trask Lane, Danvers, MA 01923, USA.

Corning Ltd., Quantum House, Marylands Avenue, Hemel Hempstead HP2 7DE, United Kingdom.

Dako UK Ltd., Denmark House, Angel Drove, Ely, Cambridgeshire CB7 4ET, UK.

Dupont Pharmaceuticals Ltd, Hertfordshire, UK

Invitrogen, Gibco and Molecular Probes products all ordered from Invitrogen:

Invitrogen Ltd, 3 Fountain Drive, Inchinnan Business Park, Paisley, UK, PA4 9RF

Promega UK, Delta House, Chilworth Science Park, Southampton, SO16 7NS, UK.

Roche Diagnostics Ltd, Bell Lane, Lewes, East Sussex BN7 1LG, UK.

Santa Cruz products ordered through Insight Biotechnology:

Insight Biotechnology Ltd, PO Box 520, Wembley, Middlesex, HA9 7YN, UK.

Sigma-Aldrich Company Ltd., The Old Brickyard, New Road, Gillingham, Dorset,
SP8 4XT.

Stressgen Products ordered through Bioquote:

Bioquote Limited, TheRaylor Centre, James Street, York, North Yorkshire, YO10
3DW, UK.

Worthington Enzymes and Biochemicals, Lorne Laboratories (distributors),
Twyford, Reading, UK.

Appendix C

Abbreviations

A1AT	α 1 Antichymotrypsin
ANP	Atrial Natriuretic Peptide
CO	Carbon Monoxide
CoCl ₂	Cobalt Chloride
CRP	C Reactive Protein
CsA	Cyclosporin A
DAB	Diaminobenzidine
DCM	Dichloromethane (Methylene Chloride)
DMSO	Dimethylsulfoxide
Grp	Glucose Regulated Protein
HO-1	Heme Oxygenase-1
HIF-1 α	Hypoxia Inducible Factor-1 α
HRE	Hypoxia Response Element
HSBP1	HSF-1 Binding Protein 1
HSE	Heat Shock Element
HSF-1	Heat Shock Factor-1
Hsp	Heat Shock Protein
IL6	Interleukin-6
iNOS	Inducible Nitric Oxide Synthase
IP	Ischaemic Preconditioning
IRI	Ischaemia Reperfusion Injury
MAPK	Mitogen Activated Protein Kinase
MTT	Methyltetrazolium
NAC	N-Acetylcysteine
NF κ B	Nuclear Factor κ B
NO	Nitric Oxide
Nrf2	NF-E2 Related Factor 2
p38	p38 MAPK
PCR	Polymerase Chain Reaction
PKC	Protein Kinase C
PP2A	Protein Phosphatase 2A

PP2C	Protein Phosphatase 2C
ROS	Reactive Oxygen Species
SA	Sodium Arsenite
siRNA	Small Interfering RNA
StRE	Stress Response Element
TNF α	Tumour Necrosis Factor α
UVC	Ultraviolet C
UW	University of Wisconsin Solution
ZnPIX	Zinc Protoporphyrin IX

HO-1 (GT)_n Genotypes

SS	Homozygous short
SL	Heterozygous
LL	Homozygous long

Appendix D

Presentations and Published Papers

Publications

The following publications have arisen from this work.

Curcumin Induces Heme Oxygenase 1 through Generation of Reactive Oxygen Species, p38 Activation and Phosphatase Inhibition.

McNally SJ, Harrison EM, Ross JA, Garden OJ, Wigmore SJ.

International Journal of Molecular Medicine 19 (1): 165-72, January 2007.

Curcumin Induces Heme Oxygenase-1 in Hepatocytes and is Protective in Simulated Cold Preservation and Warm Reperfusion Injury.

McNally SJ, Harrison EM, Ross JA, Garden OJ, Wigmore SJ.

Transplantation. 81 (4): 623-6, February 2006.

Ethical Considerations in the Application of Preconditioning to Solid Organ Transplantation.

McNally SJ, Harrison EM, Wigmore SJ.

Journal of Medical Ethics, 31 (11): 631-4, November 2005.

Optimisation of the Paired Enzyme Assay for Heme Oxygenase Activity.

McNally SJ, Ross JA, Garden OJ, Wigmore SJ.

Analytical Biochemistry, 332(2):398-400, September 2004

Presentations

Presentations arising from this work have been presented to the following learned societies.

Heme Oxygenase-1 Preconditioning Agents Differentially Modulate Hepatocyte Function. Oral Presentation at the Association of Surgeons in Training Conference, Edinburgh, March 2006.

Curcumin Induces Heme Oxygenase 1 in Hepatocytes via Nrf2, and is Cytoprotective in *In Vitro* Models of Reperfusion and Cold Storage Injury. Oral presentation at the Quincentenary Congress, Royal College of Surgeons of Edinburgh, Edinburgh 2005.

Curcumin Protects Hepatocytes via Heme Oxygenase 1 From Simulated Warm Reperfusion Injury and Following Cold Storage. Poster Presentation at the Society of Academic and Research Surgery (SARS), Newcastle, January 2005. British Journal of Surgery 92(10):1320, October 2005 (abstract)

Curcumin Preconditions Hepatocytes In Models of Warm Reperfusion Injury And Cold Storage. Oral presentation at the Scottish Society for Experimental Medicine (SSEM), Edinburgh, November 2004.

Determination of the Mechanism of Stress Protein Preconditioning by Calcineurin Inhibitors. Oral presentation at the Scottish Hospital Endowments Research Trust Open Day, Royal Society of Edinburgh, Edinburgh, June 2004.

Curcumin Induces an Atypical Stress Response in Hepatoma Cells.

Poster presentation at the Biochemistry Society Focused Meeting, London March 2004.

Curcumin modifies Heat Shock Transcription Factor-1 and induces Heme Oxygenase 1 but not inducible hsp70 in human hepatocytes Poster presentation at the British Transplantation Society Annual Congress, London, April 2003

Curcumin and the Stress Response in Human Hepatocytes.

Oral presentation at the Chiene Medal Surgical Research Meeting November 2002

Curcumin Modifies Heat Shock Factor 1 and Induces Heme Oxygenase 1 but not Hsp70 in Human Hepatocytes. Oral presentation at the Scottish Society for Experimental Medicine (SSEM), Edinburgh, October 2002.

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